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(54) Method for the microbiological preparation of human serum albumin and other heterologous proteins from a yeast.

(57) Microbiological method for the preparation of human serum albumin and other heterologous (non-yeast) proteins by growing a yeast, especially of the genus Kluyveromyces, modified by the use of recombinant DNA techniques.

EP 0 361 991 A2

METHOD FOR THE MICROBIOLOGICAL PREPARATION OF HUMAN SERUM ALBUMIN AND OTHER HETEROLOGOUS PROTEINS FROM A YEAST

The present invention relates to a microbiological method for the preparation of human serum albumin (HSA) by culturing a yeast, especially of the genus Kluyveromyces, modified by the use of recombinant DNA techniques.

HSA is a protein of 585 amino acides with a molecular weight of 66 kilodaltons (kD), taking the form of a globular, unglycosylated monomer. Its globular structure is maintained by 17 disulfide bonds which create a sequential series of 9 double loops (Brown, J.R., in "Albumin Structure, Function and Uses", Rosenoer, V.M. et al. (eds.), Pergamon Press, Oxford, (1977) 27 - 51). The genes encoding HSA are known to be highly polymorphic, and more than 30 apparently different genetic variants have been typed by electrophoretic analysis under various conditions (Weitkamp, L.R. et al., Ann. Hum. Genet. 37 (1973) 219 - 226). The HSA gene is split into 15 exons by 14 intron sequences, and comprises more than 16.9 kilobases (kb) from the putative "Cap" site to the first poly(A) addition site.

Human albumin is synthesized in the liver hepatocytes, from which it is secreted into the blood-stream. It is the most abundant protein in the blood, with a concentration of about 40 g/liter of serum; there are hence about 160 g of circulating albumin in the human body at any time. The most important role of HSA is to maintain normal osmolarity in the bloodstream. It also has an exceptional binding capacity for various substances and plays, in addition, a role in the endogenous transport of hydrophobic molecules (e.g. steroids and biliary salts) as well as of various therapeutic substances which can thus be transported to their respective action sites. Furthermore, HSA has recently been implicated in the catabolism of prostaglandins.

The synthesis of HSA in the hepatocytes first yields a precursor, prepro-HSA, which contains a signal sequence of 18 amino acids directing the nascent polypeptide to the secretory pathway. This signal sequence is cleaved off, probably by cotranslational processing, before the protein is released from the endoplasmic reticulum. This first proteolytic cleavage yields the precursor pro-HSA, which still contains a N-terminal hexapeptide (Arg-Gly-Val-Phe-Arg-Arg) not normally present in the mature form of circulating HSA.

A convertase, probably located in the Golgi vesicles removes the pro-peptide in a second proteolytic step by cleaving the hexapeptide bound to the N- terminal aspartic acid of mature HSA (Judah, J.D., and Quinn, P.L., Nature 271 (1987) 384 - 385; Bathurst, I.C. et al., Science 235 (1987) 348 - 350). However, non-processed human albumin may represent half of that present in the bloodstream in rare heterozygous individuals who carry an inherited mutation in the pro-peptide sequence, and all of the albumin in some extremely rare homozygotes (Takahashi, N. et al., Proc.Natl. Acad. Sci. USA 84 (1987) 7403 -7407). One of these variants is designated pro-albumin Christchurch and begins with the sequence Arg-Gly-Val-Phe-Arg-**Gln**$^{-1}$-Asp^{+1}, in which the substitution Arg^{-1}--> **Gln** prevents the cleavage of the pro-peptide thus mutated (Brennan, S.O. and Carrel, R.W., Nature 274 (1978) 908 - 909). Other variants known as pro-albumin Lille (Abdo, Y. et al., FEBS Letters 131 (1981) 286 - 288) and Takefu (Nagata et al., Rinsho Byori 30 (1982) 791 - 796) have the following N-terminal sequences, respectively: Arg-Gly-Val-Phe-Arg-**His**-Asp and Arg-Gly-Val-Phe-Arg-**Pro**-Asp. In each of these cases, the observed mutation affects the pair of basic amino acids Arg-Arg which normally precedes the amino-acid sequency of mature human albumin, and results from a single-base change in the arginine codon (Takahashi, N. et al., Proc.Natl. Acad. Sci. USA 84 (1987) 7403 - 7407).

Cleavage after a pair of basic amino acids is an essential feature of the maturation of other proteins, including peptide hormones, neuropeptides and plasma proteins other than HSA (Douglass, O. et al., Annu. Rev. Biochem. 53 (1984) 665 pp.). Recently, it has been disclosed that the convertase which cleaves pro-albumin to albumin in the liver is probably very closely related to the protease yscF of yeast (Bathurst, I.C. et al., Science 235 (1987) 348 - 350). This calcium-dependent thiol protease is encoded by the KEX2 gene of Saccharomyces cerevisiae, and is probably bound to the membrane of the Golgi apparatus. It is known to be involved in the maturation of the pheromone referred to as alpha-factor and in that of the killer toxin, since kex2 mutants of S. cerevisiae are incapable of cleaving the respective pro-proteins at the pair of basic amino acids Lys-Arg (Julius, D.J. et al., Cell 32 (1983) 839 pp.). Furthermore, it can be demonstrated that the yeast enzyme encoded by the KEX2 gene correctly recognizes and cleaves the normal pro-sequence (Arg-**Arg**) of albumin in vitro, but does not cleave the mutated pro-sequence (Arg-**Gln**) of pro-albumin Christchurch (Bathurst, I.C. et al., Science 235 (1987) 348 - 350). A convertase analogous to the yscF protease has recently been described as being encoded by the KEX1 gene of K. lactis (Wésolowski-Louvel, M. et al., Yeast 4 (1988) 71 - 81). In the light of this finding, the capacity of these yeasts to maturate recombinant human albumin correctly and to secrete the latter into the culture medium was tested.

Albumin accounts for 40% of the world market in plasma proteins. Commercial interest in it stems from

the fact that this product is widely used, for example, in so-called blood-volume expanders to compensate for blood losses during surgery, accidents, hemorrhages, and at doses per day and per individual in the multi-gram range.

Up to now, HSA is generally produced by conventional techniques of fractionation of plasma derived from blood donors (Cohn, E.J. et al., J. Am. Chem. Soc. 68 (1946) 459 pp), the worldwide annual consumption of plasma being in the region of 9.5×10^6 litres. It can also be extracted from human placenta according to the technique described by J. Liautaud et al. (13th Intern. Congress of IABS, Budapest; A: "Purification of proteins. Development of biological standard", Karger (ed.), Bale, 27 (1973) 107 pp.).

The development of genetic engineering and of new extraction and purification techniques has opened up the possibility of obtaining improved products of higher purity and better stability without viral contamination (e.g. hepatitis B and AIDS) and at lower cost. However, no procedure based on genetic engineering is known as yet which is efficient enough to be of economic interest for the industrial-scale production of HSA.

This is mainly due to the lack of an efficacious host/vector system which would permit the high-level production of a correctly maturated secreted albumin possessing proper tertiary structure and having the physicochemical properties of native human albumin.

Even though the use of mammalian cell cultures might appear the ideal choice for the expression of human proteins, the cost of such a procedure would be well beyond the current selling price of albumin used for pharmaceutical purposes (Klausner, A., Biotechnology 3 (1985) 119 - 125). Since this product is generally prescribed in multi-gram quantities and at a low unit price, biotechnological production of HSA seems to be feasible only with microbial fermentation systems.

Until recently, the vast majority of genetic engineering experiments have employed Escherichia coli as the host organism for the microbial production of heterologous proteins of economic importance. Even though this type of procedure has appeared to be satisfactory for a number of heterologous proteins, all attempts to produce HSA under economically acceptable conditions using this organism have been only partially successful.

One of the major drawbacks associated with the use of E. coli lies in the fact that this bacterium is, in most cases, incapable of excreting the heterologous proteins into the culture medium, and that these proteins accumulate in one of the cellular compartments. The desired protein must hence be separated from cellular matter, giving rise to complex and expensive procedures. Furthermore, E. coli produces endotoxins and pyrogens which can contaminate the proteins produced. For this reason, great care must be taken in the purification to remove residual endotoxins in the end product, especially when the latter is used as a medicinal product.

Secreted proteins often lose the property of folding correctly if they are synthesized and accumulated in the cytoplasm. In general, secretion is required in order to make possible the formation of disulfide bonds such as those present in HSA. The fact that HSA produced in E. coli is not secreted thus causes it to precipitate intracellularly in an insoluble form (Latta, M. et al., Bio/Technology 5 (1987) 1309 - 1314). Consequently, after extraction from the bacterial cells, the protein has to be denatured and then renatured in vitro.

Furthermore, E. coli lacks the cellular machinery making it possible to perform the post-transcriptional and post-translational modifications specific to eukaryotic organisms, including yeasts and humans. With regard to HSA, it appears to be difficult to induce E. coli to express at a satisfactory level the natural HSA precursor, i.e. prepro-HSA. Moreover, artifical albumins in which the HSA structural gene has been fused to secretion signals derived from the bacterial pac or ompA genes are generally processed incompletely (ibid.). Furthermore, the expression of HSA without the prepro-sequence, i.e. in the form Met-HSA, shows that the N-terminal methionine residue is not excised by the E. coli methionine aminopeptidase (MAP) (ibid.; European patent application EP 198 745, publ. 22.10.1986). In consequence, E. coli-derived HSA cannot be obtained in mature form in vivo and must be modified in vitro, by tryptic cleavage, in order to excise a phage-derived leader sequence, subsequent to denaturation and renaturation in vitro (European patent application EP 236 210, publ. 09.09.1987).

As regard other bacterial hosts, studies have recently been published on the secretion of HSA by Bacillus subtilis (Saunders, C.W. et al., J. Bacteriol. 169 (1987) 2917 - 2925). Although this study indicates that a prokaryotic organism such as B. subtilis possesses the potential for secreting high molecular weight human proteins such as HSA, it also shows that excretion in the growth medium can be obtained only when using B. subtilis protoplasts, i.e. bacterial cells whose normal cell wall has been digested by enzymatic treatment. Furthermore, the percentage of modified HSA is inversely proportional to the level of protein produced: at high expression levels, most of the recombinant HSA remains in unmaturated form (ibid.). Moreover, no data concerning the physicochemical properties of the recombinant HSA from B. subtilis have

been reported. Furthermore, the level of HSA excretion obtained by this microorganism is very low in the light of the amounts of HSA detected in the culture supernatants using immunological methods (European patent application EP 0 229 712 A2, publ. 22.07.1987).

An attractive alternative to the use of bacterial hosts for the preparation of heterologous proteins is the use of microbial eukaryotic systems such as yeasts or fungi. In effect, these organisms possess all the features of structure and cellular organization found in more complex eukaryotic organisms such as mammalian cells. In particular, yeasts are capable of carrying out the post-transcriptional and post-translational modifications which are important for the activity of many proteins. In addition, yeasts are very common in industrial-scale production, can be grown to high cell densities, are not pathogenic, do not produce endotoxins and have been used in the food industry since ancient times. Finally, in contrast to mammalian cells, genetic manipulations with yeasts are easy to perform, and classical and molecular genetics have provided a large body of data.

The term yeast is frequently used to indicate Saccharomyces cerevisiae, or baker's yeast, which is one of the most common and best known species. It is understood hereinafter that the term yeast applies to other genera, and is not restricted to the species S. cerevisiae.

The expression of prepro-HSA under the control of the chelatin promoter, has been demonstrated in S. cerevisiae at maximal levels of about 1% of total proteins (Etcheverry, T. et al., Bio/Technology 4 (1986) 726 - 730). However, the material recognized by anti-HSA antibodies which is described in this study remains cell-associated, and therefore is not truely exported into the culture supernatant. In addition, no detailed characterization of the recombinant protein has been reported.

Production of HSA in brewer's yeast using a post-fermentation process during beer brewing has also been reported (European patent application EP 0 201 239, publ. 12.11.1986). Once again, no quantitative or qualitative data concerning the product obtained has been described. Furthermore, this process leads to the expression of Met-HSA, i.e. an allele of HSA starting with the amino acid methionine just upstream from the sequence of mature albumin. The absence of a signal sequence precludes the secretion and maturation of the recombinant HSA, and causes accumulation of an intracellular albumin whose tertiary structure has not been characterized. In addition, the absence of N-terminal methionine in the final product has not been demonstrated.

The present invention describes the production of genetically engineered, modified yeast strains which can be grown in culture for mass production and which are capable of efficiently producing and excreting HSA in its native conformation into the growth medium.

A preferred expression system involves yeasts of the genus Kluyveromyces as a host and uses vectors derived from the natural K. marxianus var. drosophilarum plasmid pKD1.

Moreover, research performed with the object of producing albumin has revealed the value and importance of certain pKD1 sequences in the efficacy and especially the stability of the expression plasmids incorporating them.

The present invention hence also relates to the use of these essential elements of pKD1 in the expression of proteins other than albumin, in particular tissue plasminogen activator (tPA), metalloprotease inhibitor (TIMP) and interleukins, e.g. interleukin-1β(Il-1β), these proteins to be considered only as examples for the purpose of demonstrating that the use of pKD1 plasmid derivatives is "generic", i.e. hardly dependent, if at all, on the nature of the proteins whose expression and/or secretion is desired.

Yeasts of the genus Kluyveromyces and in particular K. marxianus (including the varieties lactis and marxianus which will hereinafter be referred to as K. lactis and K. fragilis, respectively) are important organisms and of considerable commercial interest in the biotechnology industry. K. lactis and K. fragilis are used, for example, for the commercial production of the enzyme lactase (β-galactosidase). These yeasts are capable of growth on whey, a major by-product of the dairy industry. Several Kluyveromyces strains are used for the large-scale production of "single cell protein" (SCP) which play an important role in livestock feeds. Finally, organisms of the genus Kluyveromyces are mentioned on the so-called GRAS list (Generally Recognized As Safe), which represents an important factor for the production of pharmaceutical grade products.

Gene manipulation techniques in Kluyveromyces have only recently been developed. Three types of cloning vectors have been described for this organism:

i) integrating vectors containing sequences homologous to regions of the Kluyveromyces genome and which, after being introduced into the cells, are integrated in the Kluyveromyces chromosomes by in vivo recombination. Integration, a very rare event requiring the presence of an efficient selection marker, is obtained when these vectors do not contain sequences permitting autonomous replication in the cell. The advantage of this system is the stability of the transformed strains, i.e. the fact that they can be grown in a normal nutrient support without the need for a selection pressure for maintenance of the integrated

sequences. The disadvantage is, however, that the integrated genes are only present in one or, at best, a small number of copies per cell. This low gene dosage often results in a low level of production of a heterologous protein.

ii) replicating vectors containing autonomously replicating sequences (ARS) derived from the chromosomal DNA of Kluyveromyces sp. (Das, S. and Hollenberg, C.P., Current Genetics 6 (1982) 123 - 128; International patent application WO 83/04050, publ. 24.11.1983; International patent application WO 83/04051, publ. 24.11.1983). Such vectors are of only moderate interest, since their segregation in mitotic cell division is very unhomogeneous, which results in their loss from the cells at a high frequency even when the latter are grown under selection pressure.

iii) replicating vectors derived from naturally occurring yeast plasmids, either from the linear killer plasmid pGKl-1 (k1) isolated from K. lactis (de Louvencourt, L. et al., J. Bacteriol. 154 (1982) 737 - 742; European patent application EP 0 095 986, publ. 07.12.1983), or from the circular plasmid pKD1 isolated from K. marxianus var. drosophilarum (European patent application EP 0 241 435 A2, publ. 14.10.1987). Vectors containing replication sequences derived from the linear killer plasmid are of no practical utility for mass production of heterologous proteins since a special nutrient medium is required for their maintenance and since they are lost in 40-99% of the cells in a given population after only 15 generations, even under selection pressure (European patent application EP 0 095 986, publ. 07.12.1983). The most efficient vector system for the transformation of the genus Kluyveromyces described to date is derived from the endogenous plasmid pKD1: constructions containing the entire sequence of pKD1 can be transferred with high frequency, are present in the cell in 70-100 copies and, most importantly, have a relatively high stability under non-selective conditions. Nevertheless, the usefulness of the vectors described in EP 0 241 435 remains limited to research applications inasmuch as industrial-scale fermentations require a stability of the plasmid for at least 40 generations. Even the most efficacious vector (P3) described in EP 0 241 435 is lost by about 70% of the cells in a given population after only six generations in a non-selective medium (European patent application EP 0 241 435 A2, publ. 14.10.1987). Even though it is technically feasible to maintain a selection pressure in a large-scale fermentation, the use of selective medium often results in a considerably lower cell density, and requires the use of well defined strains, rendering this approach less attractive and more expensive. Furthermore, EP 0 241 435 does not contain a single example of expression of a heterologous protein of commercial interest. In fact, the only "heterologous" gene shown to be expressed from vectors derived from pKD1 is the URA3 gene of the yeast S. cerevisiae. It hence remains to be proven that the introduction of a non-yeast gene, e.g. a gene of mammalian origin, into pKD1 and its overexpression in Kluyveromyces do not result in enhanced plasmid instability.

An object of the present invention is the production of new expression vectors capable of transforming yeasts of the genus Kluyveromyces and possessing stability characteristics markedly superior to those described in Patent Application EP 0 241 435. It will be shown that the new constructions described in the present invention are maintained at high copy numbers in 85-90% of the cells after 50 generations of growth in non-selective medium. It is thus possible to produce heterologous proteins from stable multicopy vectors using industrial strains of the genus Kluyveromyces which have been in use for many years on account of their optimal growth properties and at high cell densities.

The present invention relates, in particular, to a method for the preparation of a specified protein, in which method a yeast of the genus Kluyveromyces transformed with an expression plasmid comprising:

- genes A, B and C of plasmid pKD1,
- the inverted repeats of plasmid pKD1 (IR),
- the stability locus of pKD1,
- the origin of replication of pKD1 and an expression cassette containing a DNA encoding the structural gene for said protein under the control of sequences permitting its expression in said yeast,
- a selectable marker for the transformed yeast,
- and, optionally, an origin of replication and a selectable marker for Escherichia coli,

is cultured in a growth medium.

The high stability of the vectors described in the present invention has been achieved by exploiting fully the characteristics of plasmid pKD1. Vectors derived from pKD1 differ from all other known Kluyveromyces vectors in that they contain a specialized replication system responsible for stable plasmid maintenance at high copy number. In addition to an origin of replication this system comprises two inverted repeats, each 346 nucleotides in length and three open reading frames (genes A, B and C) which are integral parts of the plasmid (Chen, X.J. et al., Nucl. Acids Res.14 (1986) 4471 - 4481). The retention of these replication and stability elements, genes A, B and C and inverted repeats (IR) results in vectors exhibiting a very high mitotic stability. By analogy with the most extensively studied system, that of the structurally related 2 µ plasmid of S. cerevisiae, the proteins encoded by two of these genes (B and C) are probably involved in

plasmid partitioning during mitotic cell division, and might play a part in the negative regulation of gene A encoding a site-specific recombinase (FLP: Futcher, A.B., Yeast 4 (1988) 27 - 40). FLP-mediated recombination between the inverted repeats of 2 μ DNA has been shown to switch the plasmid from a regular mode of replication (one doubling of 2 μ plasmids per cell division) to a rolling circle type mode of replication (copy number amplification to about 50 copies per cell; ibid.). This change in the normal mode of replication is induced as soon as the plasmid copy number becomes too low to permit the production of sufficient quantities of gene B and C products which act as repressors of gene A encoding the FLP recombinase. By this mechanism, the copy number of 2 μ (and very probably of structurally similar plasmids such as pKD1) is maintained at high levels in an autoregulated manner and is independent of the presence of a selectable marker.

The vectors previously published in EP 0 241 435 either contain only a part of pKD1 (A15) or contain an interrupted gene A (P1 and P3 in which the PstI cloning site is located within the coding sequence of gene A (European patent application EP 0 241 435 A2, publ. 14.10.1987), thereby destroying the autoregulated replication system which is one of the characteristics of the resident plasmid pKD1. In contrast, plasmid constructions derived from pKD1 described in the present invention respect the functional integrity of all the important open reading frames of pKD1. In consequence, the stability of the plasmids described below is considerably enhanced with respect to plasmids P1 and P3, and the copy number of the new vectors is maintained at a high level throughout the cell population.

In the present invention, the term HSA will be used to denote any serum albumin of human origin or any protein isolated from yeast cells and possessing the same amino acid sequence, tertiary structure and physicochemical properties as native HSA of human origin. Furthermore, HSA variants are understood to denote natural variants, as well as molecules having the same acitivity as HSA but from which, where appropriate, the domains not essential to the activity in question have been truncated.

The present invention relates to the creation by genetic engineering of new vectors enabling HSA to be produced in a readily purifiable form using yeast cells as a eukaryotic microbial host. A feature of the production method is the efficient excretion of mature, native HSA into the growth medium of said yeasts, thereby considerably facilitating the purification of the recombinant protein. Production of HSA is obtained by growing yeasts transformed with a recombinant plasmid comprising a transcription and translation initiation region which functions efficiently in the said host and an open reading frame encoding HSA preceded by a secretion signal directing the recombinant protein to the secretory pathway of said yeasts.

According to the present invention, the new constructions include expression cassettes containing the structural gene for HSA or one of its variants. The constructions are usually prepared in a stepwise manner employing the appropriate vectors until the elements essential to expression, secretion or plasmid maintenance have been combined to form a vector which can then to introduced into the defined host(s) so as to express and excrete the desired protein. The hosts employed are of eukaryotic origin, especially yeasts, more especially of the genera Saccharomyces or Kluyveromyces and preferably the species Kluyveromyces marxianus including all its varieties, especially K. marxianus var. lactis. Thus, according to the present invention, the constructions which are prepared and described are in the nature of examples in eukaryotic organisms of microbiological origin, but are preferably directed to Kluyveromyces.

The particular hosts to be employed will preferably be industrial strains which are stable, grow to high cell densities in appropriate media and have a high level of production.

Among the strains which can be transformed with pKD1-derived plasmids, strains of the species K. wickerhamii, K. waltii and, in particular, K. marxianus var. bulgaricus (K. bulgaricus), K. marxianus var. drosophilarum (K. drosophilarum), K. marxianus var. marxianus (K. fragilis) and K. marxianus var. lactis (K. lactis) should be mentioned more especially.

The HSA coding sequence may be obtained in a variety of ways, the simplest consisting in isolating messenger RNA from human liver and synthesizing copies thereof in the form of complementary DNA (cDNA). The cloned sequences may then be modified by different methods such as in vitro site-directed mutagenesis, primer elongation, restriction, insertion of adaptors or ligation with oligodeoxynucleotide linkers. The coding sequence may be adapted, for example, to the use of preferred codons in yeast in order to optimize the efficiency of protein synthesis (translation) in the said host.

At the N-terminus of the protein sequence, a signal peptide (pre-sequence) may be introduced so as to direct the nascent protein to the secretory pathway of the host cell. This pre-sequence may correspond to the natural N-terminal leader of the protein, in particular albumin, or it may be of another origin, e.g. it may be obtained from yeast genes such as those encoding the alpha pheromone or the killer toxin.

Furthermore, a pro-sequence encoding another peptide extension may be interposed between the secretion signal sequency and the coding sequence for mature albumin. This pro-sequence is normally joined to the coding sequence by a site for cleavage by a specific protease generally involving at least two

basic amino acids, preferably Lys-Arg or Arg-Arg.

The expression cassette will comprise a transcription and translation initiation region joined to the 5′ terminus of the coding sequence, so as to direct and regulate the transcription and translation of said sequence. The choice of these promoter regions may vary according to the particular host used. These sequences are generally chosen from promoters derived from yeast genes. Of special interest are certain promoter and/or terminator regions derived from glycolytic genes of Saccharomyces or Kluyveromyces type yeast, such as the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GDP), enolases (ENO), alcohol dehydrogenases (ADH), or derived from other strongly expressed genes such as the lactase (LAC4), the acid phosphatase (PHO5), and the like. These control regions may be modified, e.g. by in vitro mutagenesis, by introduction of additional control elements or synthetic sequences or by deletions. For example, transcription-regulating elements, such as the so-called UAS (upstream activating sequences) originating from another promoter, e.g. that of the S. cerevisiae GAL10 or K. lactis LAC4 gene, may be used to construct hybrid promoters which enable the growth phase of a yeast culture to be separated from the phase of expression of a heterologous gene, depending on the carbon source chosen. A transcription and translation termination region which is functional in the intended host will be positioned at the 3′ end of the coding sequence.

The expression cassette thus constructed will be fused to one or more markers enabling the transformed host to be selected. Preferred yeast markers are dominant markers, i.e. markers conferring resistance to antibiotics such as geneticin (G418) as is the case with the expression in said yeast of the aph(3′)-I gene (aph) of the bacterial transposon Tn903, or any other toxic compound such as copper ions, inasmuch as dominant markers may be used without special host requirements. These resistance genes will be placed under the control of appropriate transcription and translation signals in the host in question. It is also possible to use markers complementing auxotrophies such as the yeast genes URA3 or LEU2.

The assembly consisting of the expression cassette and the selectable marker may be used either for transforming the host directly or it can be inserted into a replicative vector. In the case of a direct transformation, sequences homologous to regions present on a chromosome or on a resident plasmid are fused to this assembly. Said sequences will be located on each side of the expression cassette so as to induce an insertion at the homologous site by in vivo recombination. The expression cassette can also be combined with a replication system which is functional in the host in question. A preferred replication system for Kluyveromyces is derived from the plasmid pKD1, initially isolated from K. drosophilarum (Falcone, C. et al., Plasmid 15 (1986) 248 - 252; Chen, X.J. et al., Nucl. Acids Res. 14 (1986) 4471 - 4481.). A preferred replication system for Saccharomyces is derived from the yeast 2 μ plasmid. The expression plasmid can contain all or part of said replication systems or can combine elements derived from pKD1 as well as from the 2 μ plasmid. Preferred constructions are those which contain the entire sequence of the plasmid pKD1 when expression in Kluyveromyces is desired. More especially, preferred constructions are those in which the insertion site of the foreign sequences into pKD1 is localized in a 197 bp region lying between the SacI site (position 4714 of the B-form of pKD1; Chen, X.J. et al., Nucl. Acids Res. 14 (1986) 4471 - 4481) and MstII site (position 154 of the B form of pKD1; ibid.), or at one of these two sites, or alternatively at the SphI site of plasmid pKD1.

For greater convenience the plasmid can be a shuttle vector: as such, it may be transferred to a bacterial host such as E. coli, where it may be manipulated more readily than in yeast at each construction step. In this case, an origin of replication and a selectable marker functioning in the bacterial host are required. It is also possible to position restriction sites which are unique on the expression vector so that they flank the bacterial sequences. This enables the bacterial origin of replication to be eliminated by cleavage and the vector to be religated prior to transformation of the yeast cells, and this may result in a higher plasmid copy number and enhanced plasmid stability. Convenient sites such as 5′-GGCCNNNNNGGCC-3′ (SfiI) or 5′-GCGGCCGC-3′ (NotI) can, for example, be used inasmuch as they are extremely rare in yeast and generally absent from an expression plasmid. These sites may be introduced into the vector by oligodeoxynucleotide-directed mutagenesis or by adding specific oligodeoxynucleotide linkers or adaptors.

Although the present description has been written with frequent reference to human albumin, the procedure using appropriate vectors derived from the plasmid pKD1 is applicable to albumins, interleukins, tPA, TIMP and other proteins capable of benefiting from expression/excretion in said yeasts.

Among coding sequences which may be expressed, there should be mentioned, in particular,

- Il-1β
- prepro-HSA
- Met-HSA
- prepro-tPA

- Met-tPA, and
- TIMP.

Once construction of the expression vector has been completed, the latter will be introduced into the desired host. Various protocols for transformation have been described in the case of yeasts (Sherman, F. et al., "Methods in Yeast Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1986). The transformants may then be grown in a suitable medium in order to produce the desired product.

If the desired product is excreted, it may be purified in various ways from the culture supernatant. It may be cryoprecipitated, or extracted using affinity chromatography, electrophoresis or other conventional techniques. The secreted protein may be recovered from a bath culture or a continuous culture.

The examples which follow are given without implied limitation, and show special embodiment of the invention and certain advantages.

DESCRIPTION OF THE FIGURES

The diagrams of the plasmids shown in the figures are not drawn to scale, and only the restriction sites important for the constructions are shown.

Fig. 1: Construction of plasmid pXL276 containing the complete sequence encoding Met-HSA, obtained from three cDNA clones (see text) derived from poly(A)-mRNA isolated from human liver.

Fig. 2: Reconstitution of the prepro-sequence of albumin from four synthetic oligodeoxynucleotides : construction of plasmid pXL290.

Fig. 3: Construction of plasmid pXL299.

Fig. 4: Construction of plasmid pXL322.

Fig. 5: Construction of plasmid pXL855.

Fig. 6: Construction of plasmid pXL869.

Fig. 7: Nucleotide and amino acid sequence of the HindIII fragment derived from the plasmid pXL869 and containing the prepro-HSA structural gene. The heavy arrows indicate the end of the "pre" and "pro" regions.

Fig. 8: Construction of plasmid pUC-URA3.

Fig. 9: Construction of plasmid pCXJ1.

Fig. 10: Construction of plasmid pk1-PS1535-6.

Fig. 11: Construction of plasmid pUC-kan202.

Fig. 12: Nucleotide sequence of the ORF1 promoter of plasmid k1 from the ScaI restriction site and including the junction between ORF1 and the 5' region of the bacterial gene encoding aminoglycoside phosphotransferase derived from Tn903.

Fig. 13: Construction of plasmid pKan707.

Fig. 14: Stability curve of plasmid pKan707 in strain MW98-8C under non-selective growth conditions.

Fig. 15: Construction of plasmid pYG11.

Fig. 16: Construction of plasmid pYG18.

Fig. 17: Construction of plasmid pYG19.

Fig. 18: Illustration of plasmids pYG19 (prepro-HSA) and pYG23 (Met-HSA). The nucleotide sequence appearing under each plasmid is the junction between the PGK gene promoter and the structural genes for the different forms of HSA.

Fig. 19: Construction of plasmids pYG208, pYG210 (pannel **A**) and pYG221B (pannel **B**).

Fig. 20: Construction of plasmids pYG14, pYG26 and pYG29.

Fig. 21: **A**, Mutagenesis of the PGK promoter of S. cerevisiae to introduce NotI sites flanking the UAS region. The four base pairs changed by site directed mutagenesis are indicated by asterics below the NotI sites.

B, Untranslated PGK leader sequences in constructs pYG19 and pYG44. The HindIII site introduced at the -25 region of the wild-type PGK promoter by site directed mutagenesis is indicated by a box.

Fig. 22: Replacement of the UAS_{PGK} by a synthetic DNA fragment containing the UAS of the LAC4 promoter of K. lactis and reconstitution of the PGK wild-type ATG context via ligation of a synthetic adaptor to the mutagenized PGK promoter.

Fig. 23: SalI-SacI expression cassettes with various promoters and signal sequences present in pKan707 derived plasmids which direct the secretion of HSA into the growth medium.

Fig. 24: Site directed mutagenesis of the PHO5 promoter of S. cerevisae to introduce a HindIII site at position -20.

Fig. 25: Nucleotide sequence of the promoter region near the ATG initiator codon in construction

pYG51.

Fig. 26: Reconstitution of a synthetic HindIII-DraI DNA fragment containing the 5' untranslated leader sequence of the PGK promoter, the signal sequence of the killer toxin of K. lactis (pre-region) and the first few codons of the pro-HSA gene. Construction of plasmid pYG56.

Fig. 27: Schematic representation of plasmids p31/RAG2:URA3, pYG60-5 and pYG60-21.

Fig. 28: Mitotic stability of HSA expressing plasmids pYG19 and pYG23 under non-selective growth conditions: closed squares, strain MW98-8C transformed with plasmid pYG19; open squares, strain MW98-8C transformed with plasmid pYG23; open triangles, strain CBS683 transformed with plasmid pYG19.

Fig. 29: 8.5% SDS-polyacrylamide gel after staining with Coomassie Blue, demonstrating the expression and excretion of HSA from strain MW98-8C. Lanes 1-4: reference albumin extracted from human plasma (Sigma) spotted at increasing concentrations: 0.2 µg, 0.6 µg, 2 µg and and 6 µg per lane. pYG19 (prepro-HSA), pYG23 (Met-HSA) and pYG25 (control vector without expression cassette): each lane corresponds to a quantity of protein equivalent to 100 µl of the original culture. a) soluble fractions; b) insoluble fractions; c) culture supernatants.

Fig. 30: Immunoblotting of a 7.5% polyacrylamide gel blotted onto nitrocellulose as described in the text. Lane 1-5: reference albumin extracted from human plasma (Sigma) which has been dissolved in a YPD medium at various concentrations and precipitated with TCA (5% final concentration) as described. The samples of the HSA standards have hence been treated in the same manner as the culture supernatants. In both cases, the gel spots correspond to equivalent volumes. 1) precipitation experiments with a concentration of HSA of 100 mg/l; 2) 50 mg/l; 3) 25 mg/l; 4) 12.5 mg/l; 5) 6mg/l. pYG19 (prepro-HSA) and pYG23 (Met-HSA): each lane corresponds to a quantity of proteins equivalent to 20 µl of the original culture. a) culture supernatants; b) insoluble fractions; c) soluble fractions.

Fig. 31: 8.5% polyacrylamide gel after staining with Coomassie Blue, demonstrating the kinetics of excretion of HSA from strain MW98-8C.

A, lanes a-e: reference HSA (Sigma) which has been dissolved in YPD medium at various concentrations and precipitated with TCA (5% final concentration) as described (see legend of Figure 21). a) concentration of HSA (Sigma) of 6 mg/l; b) 12.5 mg/l; c) 25 mg/l; d) 50 mg/l; e) 100 mg/l. pYG19: pYG19-mediated secretion of HSA as a function of the age of the culture, 16-61 hours, as indicated. Each sample corresponds to the equivalent of 160 µl of culture supernatant.

B, lanes a-e: reference HSA (Sigma), spotted at increasing concentrations: 0.4 µg, 0.6 µg, 0.8 µg, 1.0 µg and 1.2 µg per lane. pYG19: pyG19-mediated secretion of HSA as a function of the age of the culture, 72-240 hours, as indicated. Each sample corresponds to the equivalent of 25 µl of non-concentrated culture supernatant.

Fig. 32: **A**, graphic representation of the kinetics of excretion of HSA from strain MW98-8C transformed with plasmid pYG19 and grown in an Erlenmeyer as described in the text. The concentration of albumin detected in the growth medium (mg/l) is shown as a function of the age of the culture (hours). The results of four independent experiments are shown.

B, growth curve of strain MW98-8C transformed with plasmid pYG19.

Fig. 33: Effect of carbon source on the efficiency of albumin secretion under control of the PGK or a PGK/LAC4 hybrid promoter: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of K. lactis strain MW98-8C transformed with plasmids pYG19 and pYG44.

A, Supernatants (12.5 µl per lane) from shake-flask cultures grown for 211 h either in YPD (containing 2 % glucose) (lanes 1-3) or in YPD medium to which 2 % lactose has been added after 43 h of growth (lanes 4-6). Lanes a and b: HSA Sigma 0.5 and 1.0 µg, respectively; lanes 1 and 4: plasmid pYG44-5; lanes 2 and 5: plasmid pYG44-7; lanes 3 and 6: plasmid pYG19.

B, Supernatants (25 µl per lane) from shake flask cultures grown for 113 h either in YPD (containing 2 % glucose) (lanes 1, 4 and 7), YPL (containing 2 % lactose) (lanes 3, 6 and 9) or in TYD medium to which 2 % lactose has been added after 43 h of growth (lanes 2, 5 and 8). Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 µg, respectively; lanes 1-3: plasmid pYG44-5; lanes 4-6: plasmid pYG19; lanes 7-9: plasmid pKan707 without expression cassette.

Fig. 34: Effect of promoter and signal sequence substitutions on the efficiency of albumin secretion: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of K. lactis strain MW98-8C transformed with plasmids pYG51, pYG19 and pYG58.

A, Supernatants (25 µl per lane) from shake flask cultures grown for 115 h in YPD medium to which 2 % lactose has been added after 43 h of growth. Lanes a, b and c: HSA Sigma 0.2, 0.5 and 1.0 µg, respectively; lane 1: plasmid pYG51 (PHO5 promoter); lane 2: plasmid pYG19 (PGK promoter).

B, Supernatants (25 µl per lane) from shake flask cultures grown for 66 h (lanes 1-3) or 138 h (lanes 4-6) in YPD medium. Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 µg, respectively; lanes 1, 2, 4

and 5: plasmid pYG58 (killer toxin secretion signal, two independent transformants); lanes 3 and 6: plasmid pYG19 (albumin secretion signal).

Fig. 35: Effect of carbon source on the efficiency of albumin secretion under control of the LAC4 or the PGK promoter: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of K. lactis strain CBS2359 transformed with plasmids pYG404 and pYG19.
Supernatants (25 µl per lane) from shake flask cultures grown for 166 h either in YPD (containing 2 % glucose) (lanes 1 and 4), YPL (containing 2 % lactose) (lanes 3 and 6) or in YPD medium to which 2 % lactose has been added after 48 h of growth (lanes 2 and 5). Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 µg, respectively; lanes 1-3: plasmid pYG404; lanes 4-6: plasmid pYG19.

Fig. 36: Efficiency of albumin secretion under control of an integrated versus a plasmid based PGK expression cassette: SDS-PAGE and immunoblot analysis of culture supernatants of K. lactis integrant strains MW98-8C::60-5 and MW98-8C::60-21, and of strain MW98-8C transformed with plasmid pYG19. Supernatants (25 µl per lane) were sampled from shake flask cultures grown in YPD for 137 h.

A, PAGE-SDS: Lanes a, b, c and d: HSA Sigma 0.1, 0.25, 0.5 and 1.0 µg, respectively; lane 1: strain MW98-8C transformed with plasmid pYG19; lanes 2-7: 6 independent integrant strains (MW98-8C::60-5#6, MW98-8C::60-21#9, MW98-8C::60-21#7, MW98-8C::60-5#4, MW98-8C::60-5#8, MW98-8C::60-21#11, respectively; see section E.3.7).

B, Western blot analysis: same samples as shown in A (25 µl of supernantant deposited per lane).

Fig. 37: Efficiency of albumin secretion in various Kluyveromyces strains transformed with plasmids pYG19 and pYG221B: SDS-PAGE analysis of concentrated culture supernatants stained with Coomassie blue. Cells were grown in YPD medium supplemented with G418 (200 µg/ml) for 89 h. Supernatants were concentrated ten-fold by microfiltration using centricon 30 microfiltration units (Amicon). The volume of concentrated supernatant deposited per lane was standardized as a function of biomass production and varies between 9 and 25 µl.
Lanes a and b: HSA Sigma 0.5 and 1.0 µg, respectively; lanes c and d: HSA Sigma subjected to microfiltration. A solution of HSA in YPD medium (10 mg/l) was concentrated ten-fold by microfiltration. 25 and 2.5 µl of the concentrated standard were deposited in lanes c and d, respectively. Lane 1: strain ATCC16045 (K. marxianus var. bulgaricus) transformed with plasmid pYG221B; lane 2: strain ATCC24178 (K. wickerhamii) transformed with plasmid pYG221B; lane 3: strain ATCC12424 (K. marxianus var. marxianus) transformed with plasmid pYG19; lane 4: strain ATCC56500 (K. waltii) transformed with plasmid pYG19; lane 5: strain ATCC36906 (K. marxianus var. drosophilarum) transformed with plasmid pYG221B; lane 6: strain CBS4574 (K. marxianus var. lactis) transformed with plasmid pYG19; lane 7: strain CBS683 (K. marxianus var. lactis) transformed with plasmid pYG19; lane 8: strain MW98-8C (K.marxianus var. lactis) transformed with plasmid pYG19; lane 9: strain MW98-8C (K. marxianus var. lactis) transformed with plasmid pKan707 (vector without expression cassette); lane 10: strain MW98-8C (K. marxianus var. lactis) transformed with plasmid pYG221B.

Fig. 38: Electrophoretic techniques: S = referecne HSA (Sigma), L = yeast albumin.
A - PAGE-SDS (Phast gel, Pharmacia, gradient 8-25%) staining with silver salts, S = 1 µg, L: from 0.05 to 0.25 µg.
B - Isoelectric focusing (Phast gel, pH 4.5-6.0), staining with Coomassie Blue, spots = 1 µg.
C - PAGE SDS (7.5%) staining with Coomassie Blue. S = 0.2 - 1.0 µg, L = 0.25-2.5 µg.

Fig. 39: Ion exchange chromatography - Mono Q (Pharmacia). Bottom: yeast HSA 40µg; top: standard HSA 50 µg.

Fig. 40: Reversed-phase chromatography = Nucleosil C4. a) standard HSA; b) yeast HSA.

Fig. 41: Tryptophan fluorescence emission spectrometry. Solid line: standard HSA; broken line: yeast HSA.

Fig. 42: Thermal stability at 75° C. Solid line: standard HSA; broken line: yeast HSA.

Fig. 43: Regions of the albumin recognized by the different monoclonal antibodies used for antigenic characterization.

Fig. 44: **A,B,C**: inhibition curves (see text) with respect to the nine monoclonal antibodies whose specificity is shown in Figure 43: percentage inhibition as a function of the albumin concentration (µg/ml); open squares: standard HSA; filled squares: yeast HSA.

Fig. 45: Construction of plasmid pSPGK1 and indication of the nucleotide sequence of the synthetic EcoRI fragment used as a secretion signal. The nucleotide sequence surrounding the EcoRI cloning site of plasmid pEPGK41 is also indicated.

Fig. 46: Construction of plasmid pSPGK-IL17 and indication of the nucleotide sequence corresponding to the junction between the signal sequence and Met-IL-1β.

Fig. 47: Construction of plasmid pSPGK-IL21.

Fig. 48: Construction of plasmid pSPGK-IL31.

Fig. 49: Construction of plasmid pSPHO4.

Fig. 50: Construction of plasmid pSPHO-IL14.

Fig. 51: Construction of plasmid pSPHO-IL23.

Fig. 52: Construction of plasmid pSPHO-IL35.

Fig. 53: Stability curves of plasmids pCXJ1, pSPGK-IL31 and pSPHO-IL35 in strain MW98-8C under non-selective conditions: filled squares, pCXJ1; filled triangles, pSPHO-IL35 (promoter not induced); open triangles, pSPHO-IL35 (promoter induced); open squares, pSPGK-IL31.

Fig. 54: Secretion of IL-1β in strain MW98-8C: SDS-PAGE analysis of culture supernatants stained with Coomassie blue.
Each spotted sample corresponds to 0.2 ml of supernatant of K. lactis strain MW98-8C transformed with plasmid pKan707 (control vector, lane 1), pSPGK-IL31 (lane 2) or pSPHO-IL35 (lane 3); lane 4 corresponds to 1 µg of Met-IL-1β produced in E. coli (Rhône-Poulenc Santé). The transformed cells were grown for three days in YPD medium supplemented with 200 µg/ml of G418. The supernatants were concentrated by precipitation as described in the text.

Fig. 55: Restriction map of plasmids pXL348 (Met-tPA) and pXL459 (prepro-tPA).

Fig. 56: Test of activity of the secreted tPA.
5 µl of culture supernatant of K. lactis strain MW98-8C transformed with plasmid pYG225B (prepro-tPA, lane 1), pYG224B (Met-tPA, lane 2) or pKan707 (vector, lane3) are spotted on a plate indicating tPA activity after 5 days of growth in selective YPD medium (200 µg/ml of G418).

Fig. 57: Expression and secretion of TIMP in strain MW98-8C transformed with plasmid pYG226B or pKan707.
0.25 µg of human TIMP (Rhône-Poulenc Santé, lane 1); intracellular expression of yeast recombinant TIMP (plasmid pYG226B), non-deglycosylated (lane 2) or after treatment with endoglycosidase H (lane 3); sample of E. coli recombinant TIMP (Rhône-Poulenc Santé, lane 4); intracellular fractions of strain MW98-8C transformed with the control vector pKan707: non-deglycosylated sample (lane 5) or after treatment with endoglycosidase H (lane 6); non-deglycosylated concentrated culture supernatants: strain MW98-8C transformed with plasmid pKan707 (lane 7) or PYG226B (lane 8); culture supernatants concentrated, then treated with endoglycosidase H: strain MW98-8C transformed with plasmid pKan707 (lane 9) or pYG226B (lane 10); 0.25 µg of human TIMP Rhône-Poulenc Santé, lane 11).

EXAMPLES

General cloning techniques

Classical methods of molecular biology such as cesium chlorideethidium bromide gradient centrifugation of plasmid DNA, restriction enzyme digestion, gel electrophoresis, electroelution of DNA fragments from agarose gels, transformation in E. coli, and the like, are described in the literature (Maniatis, T. et al., "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel, F.M. et al. (eds.), "Current Protocols in Molecular Biology", John Wiley & Sons, New York 1987).

The restriction enzymes were obtained from New England Biolabs (Biolabs), Bethesda Research Laboratories (BRL) or Amersham.

For ligation, DNA fragments are separated, according to size, on 0.7% agarose or 8% acrylamide gels, purified by electroelution, extracted with phenol, precipitated with ethanol and then incubated in a buffer comprising 50 mM Tris-HCL, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM ATP, pH 7.4, in the presence of phage T4 DNA ligase (Biolabs).

Where necessary, DNA fragments having 3′ recessed termini are dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIP, Pharmacia) at 37° C for 30 minutes in the following buffer: 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.5. The same procedure is used for dephosphorylation of 5′ recessed or blunt-ended termini, but the treatment is for 15 minutes at 37° C and then 15 minutes at 56° C. The enzyme is inactivated by heating the reaction mixture to 68° C for 15 minutes in the presence of 1% SDS and 100 mM NaCl followed by extraction with phenol/chloroform and ethanol precipitation.

Filling in of the 3′ recessed termini is performed with the Klenow fragment of E. coli DNA polymerase I (Biolabs). The reaction is performed at room temperature for 30 minutes in a buffer comprising 50 mM Tris-HCl, 0.4 mM dNTPs, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 µg/ml BSA (bovine serum albumin) pH 7.2. Blunt-ending of 5′ recessed termini is performed in the presence of phage T4 DNA polymerase (Biolabs) as

recommended by the manufacturer. Digestion of recessed ends is performed by limited treatment with S1 nuclease (BRL) as recommended by the manufacturer.

In vitro oligodeoxynucleotide-directed mutagenesis is performed according to the method developed by Taylor et al. (Nucleic Acids Res. 13(1985) 8749 - 8764), using the kit distributed by Amersham. Nucleotide sequencing is performed according to the dideoxy sequencing method (Sanger F. et al., Proc. Natl. Acad. Sci. USA, 74 (1977) 5463 - 5467). Enzymatic amplification of specific DNA fragments are performed by the Polymerase-catalyzed Chain Reaction procedure (Mullis K.B. and Faloona F.A., Meth. Enzyme. 155 (1987) 335 - 350; Saiki R.K. et al., Science 230 (1985) 1350 - 1354) with a "DNA thermal cycler" (Perkin Elmer Cetus) following the recommendations of the supplier.

Ligated DNA is used for transforming competents cells obtained from the following strains: E. coli MC1060 ([lacIPOZYA], X74, galU, galK, strA^r) or TG1 ([lac pro A,B], supE, thi, hsd D5/F' traD36, proA$^+$B$^+$, lacI^q, lacZ M15). Plasmid DNA is purified from ampicillin- or tetracycline-resistant transformants as appropriate. Extraction of plasmid DNA is carried out according to the procedure described by Maniatis et al. (Maniatis, T. et al., "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), which is derived from the alkaline lysis method described by Birnboim and Doly (Birnboim, H.C. and Doly, J., Nucleic Acids Res. 7 (1979) 1513 - 1523). For rapid plasmid analysis, bacterial lysates are prepared according to the method of Holmes and Quigley (Holmes, D.S. and Quigley, M., Anal. Biochem. 114 (1981) 193 - 197) and subjected to agarose gel electrophoresis analysis without prior purification. After endonuclease restriction analysis, recombinant plasmids exhibiting the desired structure are prepared on a larger scale according to the alkaline lysis procedure (Maniatis, T. et al. , "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982) using 0.5 to 1 liter cultures, and purified by cesium chloride density centrifugation.

Transformation of K. lactis with foreign DNA and purification of plasmid DNA from K. lactis are described in the text.

Example 1: CLONING OF A CASSETTE CONTAINING THE PREPRO-HSA STRUCTURAL GENE

E.1.1 Isolation of a complementary DNA (cDNA) clone encoding HSA.

The construction of recombinant plasmids permitting the expression of HSA in E. coli has been described in detail in the previous Patent Application EP 0 198 745 (European patent application EP 198 745, publ. 22.10.1986). In summary, cDNA was obtained from poly(A)mRNA isolated from human liver according to the guanidine thiocyanate procedure (Chirgwin, J.M. et al., Biochemistry 18 (1979) 5294 pp.). DNA sequence analysis permitted the isolation of three clones (pT1B11, pAA38 and p6D8, Figure 1) which contained overlapping fragments of the albumin structural gene and possessed common restriction sites within these overlaps. These sites were used in the reconstruction of a cDNA clone for HSA (Figure 1) which is complete except for its prepro-sequence.

E.1.2 Synthesis of the HSA prepro-sequence

In plasmid pXL276, the construction of which is described in detail in Patent Application EP 0,198,745, the coding sequence for mature HSA has been fused in frame to the ATG codon of the ribosome binding site (RBS) of the cII gene of bacteriophage lambda (Figure 2). This generated an NdeI site immediately upstream from the translation initiation codon of the HSA gene. pXL276 is used for reconstituting the prepro-region of HSA, which is found to be truncated in the cDNA just upstream from the TaqI site at amino acid "-1". Reconstitution is achieved by inserting a DNA fragment corresponding to the HSA prepro-sequence in the form of four oligodeoxynucleotides 33-36 bases long (Sq32, Sq34, Sq35 and Sq38; Figure 2) and a 120 bp EcoRI-NdeI fragment derived from pXL276 carrying expression signals of E. coli genes between the EcoRI and AccI sites of plasmid pUC8. The TaqI site is thus reconstituted at one end of the insert in this plasmid designated pXL290 (Figure 2). The small HindIII-TaqI fragment carrying the RBS and the prepro-sequence upstream from the TaqI site is ligated with the TaqI-PstI fragment of the HSA cDNA clone (plasmid p1B11, containing the 5' end of HSA) between the HindIII and PstI sites of pUC8 to give plasmid pXL299 (Figure 3). Plasmid pXL322 is constructed by ligating the HindIII-PvuII fragment of pXL299, carrying the RBS and the prepro-HSA sequence up to the PvuII site, with the large PvuII-EcoRI fragment of pXL276, bearing the replicon and the 3' end of the HSA coding sequence, and with the EcoRI-HindIII

fragment of pXL276 containing the promoter (Figure 4).

E.1.3 Creation of a HindIII site upstream from the HSA translation initiation codon

In order to obtain a prepro-HSA cassette capable of being readily integrated in expression vectors, the NdeI site of plasmid pXL322, described above, is changed into a HindIII site by oligodeoxynucleotide-directed mutagenesis. To this end, the HindIII-BglII fragment of pXL322 containing the 5′ end of the prepro-HSA gene is subcloned into M13mp18 and mutagenized by hybridizing the synthetic oligodeoxynucleotide 5′-ATCTAAGGAAATAC AAGCTTATGAAGTGGGT-3′ to the single-stranded template (the underlined and bold-type sequences represented the HindIII site and the translation initiation site, respectively). Plasmid pXL855 was thus obtained (Figure 5), of which the nucleotide sequence of the mutagenized region was verified by using the dideoxy sequencing method. The sequence encoding the complete prepro-HSA is reconstituted by inserting the HindIII-PvuII fragment of the mutagenized phage and the PvuII-HindIII fragment of pXL322, containing the 3′ end of the HSA structural gene, into the HindIII site of pUC8 to give plasmid pXL869 (Figure 6). This plasmid hence contains a 1.87 kb HindIII fragment containing the complete prepro-HSA structural gene as well as an untranslated 61 bp region at its 3′ end. The complete sequence of the HindIII fragment as well as the amino acid sequence of the recombinant HSA, are depicted in Figure 7. A HindIII cassette containing the Met-HSA structural gene without any secretion signal is constructed by following the same procedure except that an M13 derivative of plasmid pXL276 is mutagenized using the synthetic oligodeoxynucleotide 5′-ATCTAAGGAAATACAAGCTTATGGATGCACACAAG-3′. Reconstitution of the complete Met-HSA coding sequence in pUC8 gives plasmid pXL868, obtained in a similar manner to plasmid pXL869.

Example 2: CONSTRUCTION OF YEAST CLONING VECTORS

E.2.1 Isolation and purification of plasmid pKD1

Plasmid pKD1 may be purified from a late logarithmic phase culture of K. drosophilarum strain UCD 51-130 (U.C.D. collection, University of California, Davies, CA 95616) according to the following procedure which is derived from that described by Fleer et al. (Mol. Cell. Biol. 7 (1987) 1180 - 1192). A 1 liter culture in YPD medium (1% yeast extract, 2% Bacto-peptone (Difco), 2% glucose) is centrifuged, washed and resuspended in a 1.2 M sorbitol solution, and the cells are converted to spheroplasts in the presence of zymolyase (300 µg/ml), 25 mM EDTA, 50 mM phosphate and β-mercaptoethanol (1 µg/ml). After washing in 1.2 M sorbitol solution, the spheroplasts (corresponding to 250 ml of the original culture per tube) are resuspended in 2.5 ml of 1.2 M sorbitol and the same volume of buffer comprising 25 mM Tris-HCl, 50 mM glucose and 10 mM EDTA, pH 8.0, is added. The subsequent steps correspond to the alkaline lysis procedure already described (Maniatis, T. et al., "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), except for the precipitation of the DNA which is carried out at 23° C for 15 minutes by adding 14 ml of isopropanol. The material obtained is treated with RNase (50 µg/ml) at 37° C for 20 minutes and then with proteinase K (150 µg/ml) in a solution comprising 0.5 M NaCl, 0.5% sarkosyl and 25 mM EDTA for 1 hour at 60° C. After centrifugation in an Eppendorf microcentrifuge, the supernatants are precipitated with ethanol for 10 minutes at -70° C, the pellets are then dissolved and the DNA is purified by CsCl density-gradient centrifugation in the presence of ethidium bromide.

E.2.2 Construction of plasmid pCXJ1

The construction intermediate pUC-URA3 (Figure 8) consists of a 1.1 kb HindIII fragment containing the URA3 gene of S. cerevisiae inserted into the unique NarI site of plasmid pUC19 (Yanisch-Perron, C. et al., Gene 33 (1985) 103 - 119). The HindIII fragment is derived from plasmid pG63 (Gerbaud, C. et al., Curr. Genet. 3 (1981) 173 - 180) which has been digested with HindIII and then treated with the Klenow fragment of E.coli DNA polymerase I to generate blunt-ended termini. The 1.1 kb fragment containing the URA3 gene is purified and then inserted into the plasmid pUC19, itself cut with NarI and treated with the Klenow fragment of E. coli DNA polymerase I. Plasmid pUC-URA3 hence contains: an origin of replication for maintenance of the plasmid in E. coli, the ampicillin resistance marker for transformations in E. coli, the

lacZ gene containing a polylinker (EcoRI, SacI, KpnI, BamHI, XbaI, SalI, SphI, HindIII as unique sites) and the URA3 gene of S. cerevisiae serving as a selectable marker in uraA mutants of K. lactis.

Plasmid pCXJ1 (Figure 9) contains the complete sequence of plasmid pKD1 inserted into the unique AatII site of pUC-URA3. This construction has been obtained by linearizing pKD1 at the EcoRI site and then treating this plasmid with the Klenow fragment of E. coli DNA polymerase I. This DNA fragment is ligated with pUC-URA3, previously cut with AatII and treated with T4 DNA polymerase. Ligation of these two fragments permits reconstitution of the EcoRI restriction sites. The insertion of the DNA of pUC-URA3 at the EcoRI site of the plasmid pKD1 does not inactivate any gene required for maintaining plasmid stability and copy number (the EcoRI site being located 205 nucleotides (nt) upstream from the ATG codon of gene B; Chen, X.J. et al., Nucl. Acids Res. 14 (1986) 4471 - 4481.). In consequence, plasmid pCXJ1, which transforms K. lactis uraA cir^0 cells at high frequency, is amplified to about 70-100 copies per cell and stably maintained even in the absence of selection pressure. Due to the origin of replication contributed by pUC-URA3, plasmid pCXJ1 can also replicate in E. coli, thereby facilitating the plasmid construction and purification steps. The unique sites of plasmid pCXJ1 which can be used to insert foreign DNA and the HindIII and SalI sites from the pUC19 polylinker.

E.2.3 Construction of a fusion between the K. lactis P_{k1} promoter and the 3'-aminoglycoside phosphotransferase gene of Tn903

The use of pCXJ1 as a vector for the transformation of K. lactis and other Kluyveromyces species remains limited to strains carrying the chromosomal uraA mutation as an auxotrophic marker. In order to be able to transform industrial wild-type strains of Kluyveromyces, the 3'-aminoglycoside phosphotransferase (aph) gene of the bacterial transposon Tn903 was chosen as a dominant antibiotic resistance marker which was inserted into pCXJ1. The aph gene confers resistance to kanamycin in E. coli, and if expressed in wild-type yeast strains confers resistance to the antibiotic G418 (geneticin), which is a potent inhibitor of cell growth (Jimenez, A. and Davis, J., Nature 287 (1980) 869 -871). To permit a sufficiently strong expression of the aph gene in K. lactis, the bacterial transcription signals of the aph gene are replaced by the ORF1 promoter (P_{k1}) isolated from the killer plasmid k1 of K. lactis.

The construction of the P_{k1}-aphfusion is performed in several steps (Figures 10 to 12). In the first place, a 1.5 kb ScaI-PstI fragment of plasmid k1 is subcloned between the unique ScaI and PstI sites of pBR322; the ampicillin-sensitive and tetracycline-resistant recombinant plasmid is designated pk1-PS1535-6 (Figure 10). The 1.5 kb ScaI-PstI subcloned fragment is derived from one extremity of the linear killer plasmid; it contains the 5' half of ORF1 carried by plasmid k1 and about 220 base pairs upstream (Sor, F. and Fukuhara, H., Curr. Genet. 9 (1985) 147 - 155). Since the ScaI site is located only 22 base pairs from the left-hand extremity of k1 (Figure 10), the 1.5 kb ScaI-PstI fragment probably contains the entire promoter region of ORF1 (P_{k1}). Digestion of pk1-PS1535-6 with DdeI yields a 266 bp fragment containing 17 bp derived from pBR322 at one of its extremities (close to ScaI) and the first 11 codons of ORF1 at the other extremity. After treatment with the Klenow fragment of E. coli DNA polymerase I, the purified fragment is inserted into the unique XhoI site of plasmid pUC-kan1 (Figure 11). The latter plasmid was obtained by inserting a 1.25 kb EcoRI fragment containing the aphgene derived from Tn903 (Kanamycin Resistance Gene Block™, Pharmacia) into the unique EcoRI site of pUC19. Plasmid pUC-kan202 is obtained by performing a digestion of pUC-kan1 with XhoI, followed by a brief digestion with S1 nuclease, rendering the termini blunt-ended, followed by ligation with the DdeI fragment of pk1-PS1535-6 rendered blunt-ended with the Klenow fragment of E. coli DNA polymerase I (Figure 11). This construction enables an in-frame fusion to be obtained between the first 11 amino acids of the ORF1 gene of the linear plasmid k1 and the 5' truncated end of the aph gene of Tn903. In fact, the junction between ORF1 and aph restores the twelfth codon of aph (AGG) so that the first eleven amino acids of aph have been replaced by the first eleven amino acids of ORF1. The complete sequence of the ORF1 promoter used for the P_{k1}-aph fusion together with the beginning of the structural gene encoding aph is shown in Figure 12.

E.2.4. Construction of plasmid pKan707

Plasmid pKan707 (Figure 13) is a derivative of plasmid pCXJ1 described above. It is constructed by cutting plasmid pCXJ1 with HindIII and then treating with Klenow fragment of E. coli DNA polymerase I. The linearized plasmid with blunt-ended termini is then ligated with a 1.2 kb ScaI-HincII fragment derived from plasmid pUC-Kan202 carrying the P_{k1}-aph fusion (Figure 13). Digestion of pUC-Kan202 with ScaI and

HincII yields a blunt-ended kanamycin resistance cassette no longer containing the original bacterial promoter. The plasmid obtained, pKan707, confers resistance to very high levels of G418 (> 2.5 g/l) in K. lactis strains. As in the case of pCXJ1, plasmid pKan707 can be introduced into K. lactis cir^0 strains at high frequency, amplified to 70 - 100 copies per cell and stably maintained in the absence of selection pressure (Figure 14). The great sensitivity to G418 of most Kluyveromyces strains combined with the presence of an efficient dominant marker permitting transformation of industrial strains makes pKan707 a very useful cloning vector for yeasts of the genus Kluyveromyces.

Example 3: CONSTRUCTION OF EXPRESSION VECTORS CONTAINING THE EXPRESSION AND/OR SECRETION CASSETTES FOR ALBUMIN IN YEAST

E.3.1 Construction of expression cassettes for Met-HSA and prepro-HSA under the control of the PGK promoter of S. cerevisiae.

Plasmid pYG12 (Figure 15) contains a 1.8 kb SalI-BamHI restriction fragment consisting of the promoter and terminator regions of the PGK gene of S. cerevisiae. This fragment is derived from a 3.0 kb HindIII genomic fragment from which a 1.2 kb fragment corresponding to the structural gene has been deleted and comprising a region between the translation initiation ATG and the BglII site localized 30 codons upstream from the TAA codon specifying the termination of translation (Mellor, J. et al., Gene 24 (1983) 1 - 14). The HindIII sites flanking the 1.8 kb fragment which are thereby obtained are then destroyed and replaced by a SalI and BamHI site, respectively upstream from the promoter region and downstream from the PGK transcription terminator. A HindIII site is then introduced at the junction between promoter and terminator regions; since the site is unique, it enables heterologous genes to be readily introduced. The nucleotide sequence of this region is shown in Figure 15. The 1.8 kb HindIII fragment derived from plasmid pXL868 (see E.1.3) and encoding Met-HSA is then introduced into the HindIII site of plasmid pYG12 to give the recombinant plasmid pYG10. In a similar manner, plasmid pYG11 is generated by insertion into plasmid pYG12 of a HindIII fragment derived from plasmid pXL869 (see E.1.3) and encoding prepro-HSA (Figure 15). In consequence, two SalI-BamHI expression cassettes about 3.6 and 3.7 kb in size are thus constructed, comprising the PGK gene promoter of S. cerevisiae (P_{PGK}), followed by the gene encoding either Met-HSA or prepro-HSA, and finally the region corresponding to the transcription terminator of the PGK gene (T_{PGK}), a region including the polyadenylation site of the mRNA.

E.3.2 Construction of expression plasmids pYG19, pYG23 and pYG221.

With the object of introducing the expression cassettes described above into the vector pKan707, the 3.6 and 3.7 kb SalI-BamHI fragments derived from plasmids pYG10 and pYG11 are, in a first stage, subcloned into the corresponding sites of plasmid pIC-20R (Marsh, L. et al., Gene 32 (1984) 481 -485), to give in this manner the plasmid constructions pYG18 (prepro-HSA) (Figure 16) and pYG22 (Met-HSA). These construction intermediates give rise to P_{PGK}/Met-HSA/T_{PGK} and P_{PGK} /prepro-HSA/T_{PGK} expression cassettes in the form of SalI-SacI restriction fragments which can be directly inserted into the corresponding sites of pKan707 (Figure 17). Digestion of the latter with the enzymes SalI and SacI leads to deletion of the URA3 marker as well as that of a 35 bp fragment located upstream from gene B of pKD1. The constructions thereby obtained, designated pYG19 (prepro-HSA) (Figure 18) and pYG23 (Met-HSA) comprise the P_{PGK}/HSA/T_{PGK} expression cassettes, the virtually complete sequence of K. drosophilarum plasmid pKD1, the sequences permitting the autonomous replication of E. coli as the genes encoding β-lactamase and 3'-aminoglycoside phosphotransferase (under the control of the P_{k1} promoter), enabling, respectively, E. coli to be selected in the presence of ampicillin and K. lactis in the presence of G418.

HSA-secretion vectors retaining the URA3 gene of plasmid pKan707 have also been constructed. As shown in Figure 19A, plasmid pYG208 is derived from plasmid pYG12 by inserting a BamHI/SalI adaptor (5'-GATCCGTCGACG-3') downstream from the PGK terminator. The HindIII fragment encoding prepro-HSA is purified by electroelution from plasmid pXL869 (prepro-HSA), and cloned in the correct orientation into the HindIII site of vector pYG208 to generate plasmid pYG210; the SalI expression cassette thereby obtained (PGK promoter/prepro-HSA/PGK terminator) is purified by electroelution and cloned into the SalI site of plasmid pKan707 to generate expression plasmids pYG221A and pYG221B (Figure 19B), which only differ from one another in the relative orientation of the SalI expression cassette with respect to the vector

pKan707. In plasmid pYG221B, the orientation of the SalI expression cassette relative to the Km^r gene coding for resistance to G418 is identical to that of the SalI-SacI expression cassette carried by plasmid pYG19.

E.3.3 Construction of a PGK/LAC hybrid promoter with optimized ATG context

In order to obtain an inducible derivative of the PGK promoter present in expression plasmid pYG19, the UAS of the PGK promoter was replaced by the UAS derived from the LAC4 promoter of K. lactis - (Breunig, K.D. et al., Nucl. Acid Res. 12 (1984) 2327 - 2341). To this end the SalI-HindIII fragment of pYG12 containing the PGK promoter was cloned into bacteriophage M13mp18 giving rise to construction pYG14 (Figure 20). Using the protocol described above, two NotI sites were then introduced into the PGK promoter region via site directed mutagenesis, generating construction pYG26 (Figure 20). The synthetic oligonucleotides used for this purpose were:

5'-CAAAACCTGTGA**GCGGCCGC**TAGGACCTTG-3' (Sq417) and

5'-GG TAATTTCTTTCTC**G**A**T**AA**GCGGCCGC**GTGCTTT ATG-3' (Sq416).

Thereby a total of four basepair changes (underlined) were introduced at positions -397, -399, -536, and -541 (with respect to the ATG initiator codon defined as +1; Figure 21). The two NotI sites (bold letters) obtained in this way flank the region of the PGK promoter which has previously been described to contain two functionally distinct domains (A and M in Figure 21 A) of the UAS_{PGK} (Stanway, C. et al., Nucl. Acids Res. 15 (1987) 6855 - 6873). Digestion of this PGK derivative with the enzyme NotI allows deletion of the UAS_{PGK} and its subsequent replacement by a synthetic fragment containing the UAS_{LAC} flanked by NotI sites (Figure 22), cloned in either orientation with regard to the PGK promoter. The UAS_{LAC} fragment was obtained by hybridization and subsequent ligation of the following four oligodeoxynucleotides:

Sq526, upper strand, fragment 1:

(-448) 5'-**GGC CGC** TGT GAA AAG TGT AGC GGA AAT ATG TGG TCC GAG CAA CAG CGT CTT TTT CTA GTA GTG CGG-3' (-389)

Sq527, upper strand, fragment 2:

(-388) 5'-TCG GTT ACT TGG TTG ACA TTG GTA TTT GGA CTT TGT TGC TAC ACC ATT CAC TAC TTG AAG TCG AGT GTG A**GC**-3' (-319)

Sq528, lower strand, fragment 1:

(-319) 5'-**GGC CGC** TCA CAC TCG ACT TCA AGT AGT GAA TGG TGT AGC AAC AAA GTC CAA ATA CCA ATG TCA ACC AAG T-3' (-382)

Sq525, lower strand, fragment 2:

(-383) 5'-AAC CGA CCG CAC TAC TAG AAA AAG ACG CTG TTG CTC GGA CCA CAT ATT TCC GCT ACA CTT TTC ACA **GC**-3' (-448)

Numbers in parentheses indicate the position of each oligodeoxynucleotide with respect to the ATG codon (+1) in the wild-type LAC4 promoter (Breunig, K.D. et al., Nucl. Acids Res., 12 (1984) 2327 -2341). The region spanning positions -328 to -435 has been shown to harbour the first of three UAS elements found in the LAC4 promoter (Breunig, K.D. et al., 5th Internat. Sympos. Genet. Industr. Microorgan. (1986) Alacevic M. et al. (eds.) 551 - 560; Leonardo, J.M. et al., Mol. Cell. Biol. 7 (1987) 4369 - 4376). Letters printed in bold face represent nucleotides which are part of the two NotI sites flanking the synthetic UAS of the LAC4 gene; the junction sequence of Sq527 complementary to Sq525 is underlined.

Since the nucleotide sequence near the initiator ATG of highly expressed eukaryotic genes is thought to influence the efficiency of translation initiation (Kozak, M., Microbiol. Rev. 47 (1983) 1 - 45; Hamilton, R. et al., Nucl. Acid Res. 15 (1987) 3581 - 3593), the ATG context of the PGK promoter was modified as follows: via site directed mutagenesis an additional HindIII restriction site (bold face) was introduced at position -25 of the PGK promoter carried on pYG26, thus generating plasmid pYG29 (Figure 20). The synthetic oligodeoxynucleotide used for this experiment was 5'-TATATTTGTTGTA**AAGCTT**AGATAATTACTTCC -3' - (Sq449). The UAS_{LAC} fragment described above was then cloned into the NotI sites of pYG29 resulting in plasmids pYG63-1 and pYG63-2 (Figure 22). The PGK/LAC4 hybrid promoter regions of the latter plasmids were purified as SalI-HindIII fragments and ligated to a synthetic HindIII/BstEII adaptor composed of the following two complementary oligodeoxynucleotides: (i) 5'-AGC TTT ACA ACA AAT ATA AAA ACA **ATG** AAG TGG-3' (Sq451) and (ii) 5'-GT TAC CCA CTT CAT TGT TTT TAT ATT TGT TGT AA-3' (Sq452) (the prepro-HSA initiator codon is represented in bold letters). This adaptor reconstitutes the 22 bp immediately upstream of the wild-type PGK promoter (Figure 21 B) and comprises the first four codons of the prepro-HSA structural gene up to a naturally occuring BstEII site. The SalI-BstEII fragments obtained in this manner (carrying the PGK/LAC4 hybrid promoter with an optimized initiator ATG context) were then used to replace

the corresponding restriction fragment (harbouring the non-mutagenized PGK promoter) in construction pYG18 (c.f. Figure 16). Following this strategy two construction intermediates were obtained which only differ in the orientation of the UAS_{LAC} with regard to the PGK promoter. These new constructs were used to generate SalI-SacI expression cassettes which were cloned into the corresponding restriction sites of pKan707 (c.f. Figure 13), generating plasmids pYG44-5 and pYG44-7 (Figure 23). These plasmids are isogenic except that the synthetic NotI fragment which harbours the UAS_{LAC} is in the wild-type orientation for pYG44-5 and in the opposite orientation for plasmid pYG44-7.

E.3.4 Construction of a vector for prepro-HSA secretion under control of the PHO5 promoter of S. cerevisiae

Vector pEPHO (Figure 24) contains a 0.94 kb BamHI-PstI restriction fragment harbouring the promoter and terminator region of the acid phosphatase gene (PHO5) of S. cerevisiae. This fragment is derived from a 2.03 kb genomic BamHI-PstI fragment (Bajwa, W. et al., Nucl. Acid Res. 12 (1984) 7721 - 7739) by deletion of the PHO5 structural gene. This deletion comprises positions -3 (with respect to the ATG initiator codon +1) and +1109 (the Sau3A site located upstream of the TAG terminator codon). A polylinker EcoRI/SmaI/BamHI was inserted at the junction between promoter and terminator and can be used as cloning site for the introduction of a heterologous gene. The junction between the PHO5 promoter and this polylinker is given below (the nucleotide sequence corresponding to the polylinker is underlined):

5'-AAATTCGAGATTAGGAATTCCCGGGGATCC-3'

In order to obtain a construct in which the HSA gene is expressed under control of the PHO5 promoter and the non-translated leader region of the PGK gene, a HindIII site was introduced at position -20 of the PHO5 promoter fragment derived from plasmid pEPHO. To this end, the 0.83 kb SalI-EcoRI fragment of pEPHO was subcloned into the polylinker of bacteriophage M13mp9 and the resulting single-stranded template (pYGRF3) was then mutagenized using the oligodeoxynucleotide 5'-CCTAATCTCGAAT**AAGCTT** GCTCTATTTG-3' (Sq487; the HindIII site introduced is represented in bold characters) as mutagenic primer, resulting in plasmid pYGRF4 (Figure 24).

To facilitate the construction of a variety of expression cassettes containing different promoters and/or terminators, a derivative of plasmid pIC-20R (c.f. Figure 16) was generated in which the HindIII site of the polylinker was destroyed by treatment of the cohesive ends with the large fragment of polymerase I (Klenow) of E. coli. This new cloning vector (pIC-20RΔH3) was then used to introduce the SalI-BamHI fragment of plasmid pYG12 (c.f. Figure 15) containing the promoter and terminator regions of the PGK gene, thus generating plasmid pYG61. By using promoter modules as SalI-HindIII fragments and terminator modules as HindIII-BamHI fragments, expression cassettes can be generated with any combination of transcriptional initiation and termination signals into which the structural gene to be expressed has been cloned as a HindIII restriction fragment. Digestion of these construction intermediates with the enzymes SalI and SacI allows subsequent introduction of the new expression cassettes into the corresponding sites of yeast cloning vector pKan707 (c.f. Figures 17 and 18).

Following this basic strategy, the SalI-HindIII PGK promoter fragment of plasmid pYG61 was replaced by the SalI-HindIII fragment of pYGRF4 harbouring the PHO5 promoter. Insertion of the prepro-HSA structural gene isolated from plasmid pYG44-5 as HindIII fragment (c.f. Figure 23) and transfer of the resulting expression cassette as SalI-SacI fragment into plasmid pKan707 give rise to secretion vector pYG51 (Figure 23). This plasmid thus contains the PHO5 promoter of S. cerevisiae up to position -17 (including all transcription initiation sites (Rudolph, H. and Hinnen, A., Proc. Natl. Acad. Sci. USA 84 (1987) 1340 - 1344), Figure 25), followed by 21 bp of the untranslated PGK leader sequence, the prepro-HSA structural gene, and finally the PGK terminator.

E.3.5 Construction of a vector for HSA secretion under control of the LAC4 promoter of K. lactis

Using the polymerase-catalyzed chain reaction (PCR) technique, the LAC4 promoter of K. lactis was amplified from yeast genomic DNA as a SalI-HindIII restriction fragment. Total genomic DNA isolated from strain CBS2359 served as template and the following synthetic oligodeoxynucleotides were used as primers for the Taq-polymerase reaction (the SalI and HindIII sites introduced upstream and downstream, respectively, of the LAC4 promoter of K. lactis are represented in bold letters):

1) 5'-CCC**GTCGAC**ATGTAGAGTAGACAACAGACAGGGAGGGC-3'

2) 5'-A**AAGCTT**ATCTTTCAGTTCTCGATGAGTATGTGTGTT-3'

The design of these primers was based on the LAC4 sequence previously published by Leonardo J.M. et al. (Mol. Cell. Biol. 7 (1987) 4369 - 4376). The SalI-HindIII restriction fragment obtained in this manner contains the LAC4 promoter region spanning positions -1198 to -1 (the ATG initiator codon being defined as +1). The amplified material was digested with the enzymes SalI and HindIII, cloned into plasmid pYG61 (c.f. section E.3.4), and its nucleotide sequence was confirmed using the dideoxy chain termination method.

Following the general scheme described in the previous section a pKan707 derivative was obtained, designated pYG404 (Figure 23), in which the prepro-HSA gene is expressed under the control of the lactose-galactose inducible LAC4 promoter of K. lactis. The sequence immediately upstream of the HSA initiator codon in this specific construction is derived from plasmid pYG44-5 (fragment HindIII) and therefore contains part of the untranslated leader of the PGK gene (c.f. section E.3.3).

E.3.6 Construction of a vector for HSA secretion under control of the PGK promoter of S. cerevisiae and the killer toxin secretion signal of K. lactis

Using two pairs of complementary synthetic oligodeoxynucleotides we reconstituted a HindIII-DraI fragment which contains the following elements: (i) the ATG proximal 21 bp of the PGK promoter, (ii) the secretion signal sequence ("pre"-region) of the killer toxin gene from plasmid k1 of K. lactis (Stark M.J.R. and Boyd A., EMBO J. 5 (1986) 1995 - 2002), and (iii) the first 17 amino acids of the pro-HSA gene up to the N-terminal DraI site, fused in frame to the killer toxin pre-region. The four oligodeoxynucleotides used for this construction were as follows:

Sq1000, upper strand, fragment 1:
5'-**AGCTT**AGCTTTACAACAAATATAAAAACAATGAATATATTTTACATATTTTTGTTTTTGCTGTC-3'
Sq998, upper strand, fragment 2:
5'-ATTCGTTCAAGGTAGGGGTGTGTTTCGTCGAGATGCACACAAGAGTGAGGTTGCTCATCGG**TTT**-3'
Sq996, lower strand, fragment 1:
5'-AAAAACAAAAATATGTAAAATATATTCATTGTTTTTATATTTGTTGTAAAGCT**A**-3'
Sq997, lower strand, fragment 2:
5'**AAA**CCGATGAGCAACCTCACTCTTGTGTGCATCTCGACGAAACACACCCCTACCTTGAACGAATGACAG-C-3'

Letters printed in bold face represent nucleotides which are part of the HindIII and DraI sites used in this experiment; the junction sequence of Sq998 complementary to Sq996 is underlined.

After phosphorylation of oligodeoxynucleotides Sq996 and Sq998, a double stranded HindIII-DraI fragment was generated according to the scheme depicted in Figure 26. This 130 bp fragment was purified by gel electrophoresis and ligated to a 1.04 kb DraI-XbaI fragment containing part of the HSA structural gene isolated from plasmid pXL869 (c.f. Figure 6). The HindIII-XbaI fragment obtained in this manner was then cloned into bacteriophage M13mp10, generating construction pYG56 (Figure 26). The correct nucleotide sequence of this intermediate was confirmed by dideoxy sequencing.

The entire prepro-HSA structural gene can then be reconstituted by ligation of the following three elements: (i) the 1.17 kb HindIII-XbaI fragment derived from the pYG56 construct just described, (ii) the XbaI-BamHI fragment of pYG18 (c.f. Figure 16) containing the carboxy-terminal half of the HSA gene as well as the PGK terminator region, and (iii) a BamHI-HindIII fragment of plasmid pYG62. This latter plasmid is a derivative of construction pYG61 (c.f. section E.3.4) in which the SalI-HindIII fragment carrying the PGK promoter has been replaced by the equivalent restriction fragment derived from plasmid pYG29 (c.f. Figure 20). The BamHI-HindIII fragment of plasmid pYG62 used in this construction thus contained the E. coli origin of replication, the ampicillin resistance gene, and the PGK promoter with a HindIII site introduced at position -25 (c.f. section E.3.4). The resulting pIC derivative was designated pYG57. This latter plasmid was used as source of a SalI-SacI fragment which was cloned into the corresponding sites of yeast cloning vector pKan707 giving rise to expression vector pYG58 (Figure 23).

E.3.7 Construction of an integrative vector for directing a PGK/prepro-HSA secretion cassette to the RAG2 locus of K. lactis

Plasmid p31/RAG2:URA3 (Figure 27), kindly provided by Dr. M. Wésolowski-Louvel (Institut Curie, Orsay, France), contains a 2.3 kb genomic NheI fragment harbouring the promoter and N-terminal region of the RAG2 structural gene of K. lactis which most likely encodes a phosphoglucose isomerase (Wésolowski-Louvel M. et al., Nucl. Acid Res., 16 (1988) 8714). Plasmid p31/RAG2:URA3 was constructed by insertion of

a 1.6 kb EcoRI fragment derived from plasmid pCXJ1 (c.f. Figure 9), harbouring the URA3 gene of S. cerevisiae, into the EcoRI site of the RAG2 promoter. The 3.9 kb NehI fragment obtained in this way was then cloned into the unique NheI site of plasmid pBR322.

By cutting plasmid pYG18 (Figure 16) with the restriction enzymes EcoRV and SmaI a 3.7 kb fragment was obtained containing the same PGK prepro-HSA expression cassette as the one used in yeast secretion vector pYG19 (Figure 18). Insertion of this cassette into the unique SmaI site of plasmid p31/RAG2:URA3 (located 3' of the URA3 marker gene) generated constructions pYG60-21 and pYG60-5 (Figure 27) which only differ from each other with respect to the orientation of the prepro-HSA expression cassette.

By cutting plasmids pYG60-21 and pYG60-5 with the enzyme NheI a 7.7 kb fragment was generated containing the HSA expression cassette and the URA3 marker gene flanked by RAG2 derived sequences. These latter sequences served to target the integration of the entire fragment to the RAG2 locus of K. lactis strain MW98-8C via homologous recombination (Rothstein R.J., Methods in Enzymol. 101 (1983) 202 - 211). Integration at the RAG2 locus was confirmed by Southern analysis for three out of six integrants tested (MW98-8C::60-5 integrant clone #6 and MW98-8C::60-21 integrants clones #7 and 9). The remaining three integrants were found to carry one (MW98-8C::60-5 integrant clone #8 and MW98-8C::60-21 integrant clone #11) or two (MW98-8C::60-5 integrant clone #4) HSA expression cassettes integrated at loci other than RAG2. All six of the confirmed integrants were tested for efficiency of HSA secretion.

Example 4: TRANSFORMATION OF KLUYVEROMYCES SPECIES WITH PLASMIDS EXPRESSING HSA

E.4.1 Transformation procedures

Transformants of K. lactis strain MW98-8C (α uraA arg lys $K^{\oplus}$ pKD1$^{\oplus}$) were obtained either by the spheroplast formation technique initially described by Hinnen et al. (Hinnen, A. et al., Proc. Natl. Acad. Sci. USA 75 (1978) 1929 -1933) and appropriately adapted, or by treating the whole cells with lithium acetate (Ito, H. et al., J. Bacteriol. 153 (1983) 163 - 168), which favors DNA incorporation in the presence of polyethylene glycol (PEG 4000, Sigma). Transformants of all other Kluyveromyces strains described below were exclusively obtained by treatment with lithium acetate.

The modifications relating to spheroplast formation have already been described (Bianchi, M. et al., Curr. Genet. 12 (1987) 185 - 192). When the method involving lithium acetate is used, cell growth is performed at 28° C in 50 ml of YPD medium with agitation, and to an optical density at 600 nm (OD_{600}) between 0.6 and 0.8. The cells are harvested by low-speed centrifugation, washed in sterile TE solution (10 mM Tris-HCl pH 7.4, 1 mM EDTA), resuspended in 3-4 ml of lithium acetate solution (0.1 M in TE) to give a cell density of about 2×10^8 c/ml and then incubated at 30° C for 1 hour with moderate agitation.

0.1 ml aliquot portions of the resulting suspension of competent cells are incubated at 30° C for 1 hour in the presence of DNA and PEG 4000 at a final concentration of 35%. After a thermal shock of 5 minutes at 42° C, the cells are washed twice with sterile water, resuspended in 0.2 ml of sterile water and transferred to 10 ml tubes. YPD medium containing 0.7% agar and kept molten at 46° C is then added (5ml) and the mixture is immediately poured onto YPD plates. After solidification, an additional overlayer of 5 ml of top agar is then added. After a 18 to 24 hours incubation at 28° C, 0.16 ml of G418 solution (Geneticin 50 mg/ml, GIBCO, Grand Island, N.Y.) is spread onto the plates and transformants are counted after 4-5 days of additional incubation at 28° C.

Direct plating of the cells on YPD + G418 plates (i.e. omitting the use of top agar) results in the appearance of clones of larger size for the transformed K. lactis cells. However, a lower concentration of G418 (final concentration 50 µg/ml) has to be used to observe colonies, which leads to the selection of mutants partially resistant to G418 and derived from non-transformed cells. In addition, the transformation efficiency is at least ten times lower than the efficiency observed after phenotypic expression of the resistance gene for 18 to 24 hours.

E.4.2 Mitotic stability of HSA-expressing pKD1 derived plasmids under non selective growth conditions

The mitotic stability of plasmids pYG19 and pYG23 is measured at different time intervals after growth in nonselective medium; it is determined as the ratio between the final and the initial percentages of cells growing on YPD plates containing 200 µg/ml of G418. As shown in Figure 28, both plasmids are

exceptionally stable despite the high level of expression of a heterologous gene. When transformants of strain MW98-8C are grown in a non-selective medium for 40 cell generations, 40-45% of the cells have maintained these plasmids. Plasmid pYG19 shows even higher mitotic stability in K. lactis strain CBS683: over 80 % of the cells maintain the plasmid after 40 generations of non-selective growth.

E.4.3 pYG19 and pYG23 transformants of K. lactis MW98-8C: HSA expression and secretion

The expression and secretion levels of K. lactis MW98-8C cells containing plasmids pYG19 (prepro-HSA) and pYG23 (Met-HSA) are determined after different time intervals after growth at 28° C in non selective YPD medium and with constant agitation. The culture supernatants are obtained by two consecutive centrifugations (5 minutes at 4000 rpm in a Kontron Hermle Z-365 K centrifuge) and then 10 minutes at 12000 rpm) in order to eliminate all possible contamination by cells. A sample of the second supernatant (0.5 ml) is heated to 95° C for 15 minutes in presence of an equal volume of sample buffer containing 0.125 M Tris-HCl, 20% glycerol, 10% 2-mercaptoethanol (β-ME), 4.6% sodium dodecyl sulfate (SDS) and 0.4% bromophenol blue. When a concentration of the proteins present in the supernatant is desired, 0.4 ml of a 100% w/v trichloroacetic acid solution (TCA) is added to 8 ml of supernatant and the proteins are precipitated on ice for 1 hour. The precipitated material is recovered by centrifugation for 20 minutes at 15000 rpm and then resolubilized by heating to 95° C for 15 minutes in 0.5 ml of sample buffer containing 63 mM Tris-HCl, 10% glycerol, 5% β-ME, 2.3% SDS and 0.2% bromophenol blue.

The intracellular expression of albumin is detected using cell extracts prepared as follows: the equivalent of 0.25 ml of a cell pellet (washed once in saline buffer) is resuspended in the same volume of lysis buffer kept on ice and containing 67 mM phosphate, pH 7.5 mM β-ME, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µM leupeptin and 2 µM pepstatin A (Sigma). After addition of 0.5 ml of zirconium beads (0.5 mm diameter) stored in phosphate buffer (67 mM, pH 7) at 4° C, the cells are ruptured by 7 consecutive 30 second periods with a cell disrupter (Biospec Mini Bead-Beater, Biospec Products) interspersed by 2 minute cooling periods on ice. In these conditions, the efficiency of cell disruption is more than 80% as judged by counting the cells under a phase-contrast microscope. The liquid fraction bereft of zirconium beads is transferred to an Eppendorf tube and the beads are washed 3 times with 0.2 ml of lysis buffer and then collected in the same Eppendorf tube. By centrifugation of the tube for 15 minutes at 4° C and at 12000 g, a soluble protein fraction (supernatant) and an insoluble protein fraction (pellet) are thus defined. These two fractions are taken up to give the same final volume of sample buffer. These samples are then heated for 15 minutes to 95° C and aliquotes are layered on an 8.5% SDS-polyacrylamide gel preceded by a 5% stacking gel (Laemli, U.K., Nature 227 (1970) 680 - 685), and subjected to a current of 25 mA until the bromophenol blue reaches the bottom of the gel.

Figure 29 shows the result of a typical experiment which enables an assessment to be made of the expression and secretion of albumin obtained with K. lactis strain MW98-8C transformed with plasmids pYG19 (prepro-HSA), pYG23 (Met-HSA) and pYG25 (vector devoid of an expression cassette), after growth in 50 ml of YPD medium without G418 at 28° C for 68 hours. Each sample corresponds to 100 µl of original culture and enables the soluble fractions, insoluble fractions and culture supernatants to be compared after migration in 8.5% polyacrylamide gel and Coomassie Blue stain. A protein band which comigrates with commercial human albumin (Sigma) serving as a molecular weight reference, is detectable in the samples derived from the cells transformed with plasmids pYG19 or pYG23, but not with cells containing the vector pYG25 which does not contain the HSA gene. It will be noted that, whereas virtually the whole of the albumin expressed using pYG19 (prepro-HSA) is exported into the culture supernatant, all the albumin produced using plasmid pYG23 (Met-HSA) is present in the insoluble cytoplasmic protein fraction. In addition, the results in Figure 29 show that albumin excreted by the cells containing plasmid pYG19 is the only extracellular protein present in significant amounts. In the supernatant of a shake flask culture of MW98-8C transformed with plasmid pYG19 and grown in appropriate conditions (c.f. section E.4.6.), the albumin concentration can reach 150 mg HSA/liter.

E.4.4 Immunological detection of the albumin produced by K. lactis.

The identity of the yeast proteins which co-migrate with albumin standard may be tested by immunological detection. To this end, a polyacrylamide gel is blotted onto a nitrocellulose filter (Schleicher and Schuell, 0.45 µm) using a semi-dry blotting apparatus (Biometra) in a transfer buffer containing 25 mM Tris base, 150 mM glycine and 10% methanol. The blotting time is 30 minutes using a current of

approximately 0.85 mA/cm² of gel surface. After blotting, the filter is incubated for three times 5 minutes with 50 ml of buffer A (5% skimmed milk powder, 0.2% Tween 20 in PBS buffer [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4]), followed by incubation for 30 minutes in 40 ml of buffer A containing rabbit polyclonal antibodies directed towards HSA and diluted 1:1000. After 3 rinses of the filter with buffer A, a second, biotinylated antibody recognizing the rabbit antibodies (Vectastain ABC Immuno Peroxidase Kit, Vector Laboratories), is added on the basis of one drop per 50 ml of buffer A. After incubation of the filter for 30 minutes, the filter is rinsed 3 times with buffer B (0.2% Tween 20 in PBS) and then incubated in the presence of an avidin DH/biotinylated peroxidase H complex. The avidin/biotinylated peroxidase complex is prepared immediately before use by diluting 1 drop of each of reagents A and B of the kit in 5 ml of buffer B after incubation at 23° C for 30 minutes. The filter is incubated in a 1:10 dilution of the complex in buffer B (50 ml in total) for 30 minutes. The filter is rinsed again with buffer B for 3 5-minute periods and developed for 2-3 minutes in a solution of 10 ml containing 0.02% H_2O_2 and 10 ml of a solution containing 1 mg/ml of diaminobenzidine and 0.4 mg/$NiCl_2$ in 0.1 M Tris-HCl pH 7.4. Figure 30 shows that K. lactis MW98-8C expresses (pYG23) and excretes (pYG19) a protein recognized by anti-HSA polyclonal antibodies. This antigenic material is specifically associated with the presence of an albumin-expressing cassette since it is not detected in cell extracts or culture supernatants from yeasts transformed with the vector without an expression cassette.

E.4.5 Kinetics of HSA secretion

As shown in Figure 31A, the secretion of albumin takes place with relatively slow kinetics in an Erlenmeyer. The maximum excretion levels appear to take place after 70 to 100 hours of incubation of a culture originally inoculated at 10^5 cells/ml (Figure 31B). No significant increase or decrease is detectable between 100 and 240 hours of culture, suggesting a good stability of HSA under these growth and temperature conditions. Since there is no accumulation of proteolytically degraded material during this period, it may be concluded that no extracellular protease which might degrade the excreted albumin is present.

A graphic representation of the kinetics of excretion (A), as well as the growth curve of the strain MW98-8C transformed with plasmid pYG19 (B) is shown in Figure 32; these results show that the excretion of albumin continues after the cells have reached the stationary growth phase. This observation is in agreement with observations relating to other expression systems in yeast (Tschopp, J.F. et al., Bio/Technology 5 (1987) 1305-1308).

E.4.6 Efficiency of HSA secretion with plasmids pYG44, pYG51, pYG404, and PYG58.

The efficiency of HSA secretion has been tested under control of the PGK promoter of S. cerevisiae - (plasmids pYG19; Figures 29 to 32), a PGK/LAC4 hybrid promoter with optimized ATG context (plasmids pYG44-5 and pYG44-7; Figure 33), the PHO5 promoter of S. cerevisiae (plasmid pYG51; Figure 34 A), and the LAC4 promoter of K. lactis (plasmid pYG404; Figure 35). In addition, the secretion signal sequence (pre-region) of the killer toxin of K. lactis has been fused in frame to the pro-HSA structural gene (plasmid pYG58; Figure 34 B) and the secretion efficiency of the fusion protein compared to that of the natural prepro-HSA (for details of plasmid constructions c.f. example 3). The results obtained with these constructions can be summarized as follows:

The level of HSA secreted into the culture medium when expressed from the PGK/LAC4 hybrid promoter present in plasmid pYG44 is increased two to three fold if cells are cultured in glucose containing media to which lactose is added between mid-log and early stationary growth phase (Figure 33). This effect is not observed if the addition of lactose is replaced by an equivalent amount of glucose (data not shown). If cells are cultured in media containing lactose as sole carbon source, levels of secreted HSA are not higher as the ones obtained with glucose containing media (Figure 33 B). The lactose mediated increase of HSA secretion observed after "induction" of cells subsequent to initial cultivation in glucose, however, is not dependent on the presence of the UAS element of the LAC4 promoter of K. lactis. This lactose mediated increase in HSA secretion is equally found when HSA expression is directed by the PGK promoter (Figure 33 A, B for strain MW98-8C transformants; Figure 35 for CBS2359 transformants) or the PHO5 promoter of S. cerevisiae (data not shown).

HSA secretion under control of the LAC4 promoter of K. lactis results in lower HSA levels as the ones obtained with the PGK promoter of S. cerevisiae (Figure 35). On the other hand, LAC4 directed HSA

expression is tightly regulated: no secreted HSA could be detected in culture supernatants of CBS2359 transformants when glucose was the only available carbon source (Figure 35).

Surprisingly, when cells are grown in complete medium (YPD), the efficiency of PHO5 directed HSA secretion is only slightly below the one observed with constructs containing the glycolytic PGK promoter (Figure 34 A).

The replacement of the HSA secretion signal by the pre-region of the killer toxin of K. lactis does neither influence the kinetics nor the efficiency of HSA secretion (Figure 34).

E.4.7 Efficiency of HSA secretion with integrated expression cassettes.

The PGK/prepro-HSA expression cassette present in plasmid pYG19 has been integrated into the K. lactis chromosomal DNA by the one step gene disruption method (for experimental details c.f. section E.3.7). As shown in figure 36, the levels of HSA secreted into the culture medium by the integrant strains (lanes 2-7) are at least twenty fold below those obtained by the pKD1 derivative pYG19 (lane 1). HSA secretion was basically the same whether integration of the expression cassette occured at the RAG2 locus or elsewhere in the K. lactis genome (Figure 36). Furthermore, the orientation of the expression cassette with regard to the URA3 selectable marker gene did not have a significant influence on HSA secretion levels (Figure 36, lanes 2 and 3). However, a twofold increase of secreted HSA was observed with transformants which contained two integrated expression cassettes per genome (Figure 36, lane 5). In summary, the efficiency of HSA secretion appears to be directly linked to the number of copies of the structural gene carried in a transformed yeast cell.

E.4.8 Secretion of HSA in a variety of species and strains of the genus Kluyveromyces.

Plasmids pYG19, pYG221B or pKan707 have been used to transform the following strains of Kluyveromyces: ATCC16045 (K. marxianus var. bulgaricus), ATCC24178 (K. wickerhamii), ATCC12424 (K. marxianus var. marxianus), ATCC56500 (K. waltii), ATCC36906 (K. marxianus var. drosophilarum), CBS4574 (K. marxianus var. lactis), CBS683 (K. marxianus var. lactis), and MW98-8C (K. marxianus var. lactis). Detectable levels of secreted HSA were obtained with all transformants examined, even though appreciable variations were observed among different strains. The highest levels were found using transformants of strains MW98-8C (CBS579.88) and CBS683, the lowest level was observed with strain ATCC16045 where it could only be detected using immunological methods (data not shown). Plasmids pYG19 and pYG221B mediated comparable levels of secreted HSA in all strains tested.

Example 5: PURIFICATION OF HSA SECRETED INTO THE GROWTH MEDIUM

In a typical purification experiment, strain MW98-8C transformed with plasmid pYG19 is grown in YPD medium for 72 hours under the standard conditions already described. A culture supernatant (0.5 liter) is freed from all cellular contamination by centrifugation, and incubated at 4° C for 15 minutes in the presence of 60% ethanol (V/V). The precipitate is recovered by centrifugation, redissolved in 10 ml of a solution comprising 50 mM Tris-HCL pH 8.0, and then loaded onto a Trisacryl blue column (I.B.F., France). The recombinant albumin is eluted from this column with 3M NaCl solution. After dialysis of the fractions containing HSA against 50 mM Tris-HCl, these fractions are purified on MONO Q column (Pharmacia), and eluted at a NaCl concentration of 0.25 M. In a final stage, chromatography on Superose 12 (Pharmacia) enables the HSA to be obtained at more than 99% purity as judged from the silver staining of SDS polyacrylamide gels.

Example 6: CHARACTERIZATION OF THE SECRETED AND PURIFIED ALBUMIN

The lack of a test enabling the biological properties of albumin to be measured in vitro does not provide an easy mean to assess the quality of the recombinant albumin. For this reason, after purification, the albumin excreted by K. lactis is hence characterized by several physicochemical and immunological tests. These tests demonstrate that the recombinant albumin is secreted by K. lactis in a mature form and in its native configuration: it is not distinguishable from human serum albumin extracted from plasma with respect

to any of the criteria applied.

E.6.1 PAGE-SDS: Coomassie Blue and Silver staining.

Electrophoresis is performed on a SDS polyacrylamide gel (7.5%) (Figure 38 C) as described above, or by using the"Phast gel" system (Pharmacia, Figure 38 A). Different amounts of recombinant albumin were compared with a commercial preparation of standard albumin (Sigma) extracted from human plasma. Staining of the gel with Coomassie Blue (Figure 38 C) or with silver salts (Figure 38 A) shows the complete homogeneity of the albumin samples from yeast.

E.6.2 Isoelectrofocusing.

Isoelectric focusing is performed between pH 4.5 and 6.0 (Phast gel, Pharmacia, Figure 38 B), and between pH 4.0 and 7.0 (Immobiline, LKB). The isoelectric point of the recombinant HSA is identical to that of the reference human HSA (pI = 4.8).

E.6.3 Native PAGE and immunoblotting.

Electrophoresis of the recombinant HSA in a non-denaturing polyacrylamide gel (10%), followed by blotting onto a nitrocellulose filter and immunodetection under the conditions described in section E.4.4, reveal a comigration with the standard albumin extracted from human plasma. This suggests very strongly that correct maturation of the recombinant prepro-HSA takes place during the process of secretion in K. lactis . No contamination with non-maturated HSA is detectable, which implies that cleavage of the signal sequence and of the pro-sequence of HSA takes place efficiently in this yeast.

E.6.4 Molecular sieving chromatography

Molecular sieving chromotography is performed with Superose 12 (Pharmacia). The flow rate of the elution buffer (50 mM Tris-HCl pH 8.0) is maintained at 1 ml/minute. The concentrations of recombinant albumin and of reference albumin are 0.4 and 1 mg/ml respectively. The elution of albumin is detected by measuring the absorbance at 280 nm. In both cases, the elution volume is identical (11.5 ml).

E.6.5 Anion exchange chromatography

The recombinant albumin is eluted from a Mono Q HR 5/5 column (Pharmacia) at a concentration of 0.31 M NaCl, as is the reference albumin derived from human plasma. Figure 39 shows the chromatographic profiles obtained for injections of 100 µl of recombinant HSA (0.4 mg/ml) and of reference HSA (0.5 mg/ml) using a column equilibrated with 50 mM Tris-HCl pH 8.0, and at a constant flow rate of 1 ml/minute.

E.6.6 Reversed-phase chromatography

The behavior of the yeast albumin in reversed-phase is analyzed on a Nucleosil (C4) column with buffers A (water containing 0.1% trifluoroacetic acid (TFA) and B (acetonitrile containing 0.1% TFA). Figure 40 shows the elution, with a gradient of 20 to 80% of B in A, of the yeast albumin, which exhibits the same retention time as the reference albumin.

E.6.7 Amino acid composition

The amino acid composition of the recombinant HSA is determined by reversed-phase chromatography after acid hydrolysis (6 N HCl) and phenylisothiocyanyl derivatization. The results obtained by this method show clearly an identical composition to that of the reference albumin derived from human plasma.

E.6.8 N-Terminal sequence

The use of an automated apparatus for Edman degradation (Applied Biosystems) shows that the N-terminal sequence of the HSA secreted by K. lactis is Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys-Asp-Leu-Gly. The determination of this sequence hence confirms the results derived from the native polyacrylamide gel electrophoresis experiments, and demonstrates the correct and complete maturation of the albumin secreted by K. lactis.

E.6.9 Tryptophan fluorescence

After excitation at 295 nm, the fluorescence emission of the single tryptophan shows the same profile for both recombinant and plasma albumins (maximum at 337 nm), indicating an identical hydrophobic environment around this amino acid (Figure 41).

E.6.10 Thermal stability

The reference albumin (Sigma) and the albumin secreted by the yeast have identical stability kinetics at 75° C (Figure 42), which suggests that the bonds stabilizing the protein structure (hydrophobic interactions and disulfide bonds) are identical in both cases.

E.6.11 Reactivity towards monoclonal antibodies

The antigenicity of the recombinant albumin was studied with different monoclonal antibodies directed towards human albumin. Figure 43 shows their specificity: the antibodies HA10, HA11 and HA13 correspond to epitopes whose conservation requires the integrity of the whole molecule. The antibodies HA21, HA6, HA4, HA3, HA8 and HA1 correspond to epitopes localized along the peptide chain from the N-terminal to the C-terminal end (Doyen, N. et al., Mol. Immun. 22 (1985) 1 - 10; Lapresle, C., Anal. Biochem. 174 (1988) 308-312).

The test employed is an ELISA (Enzyme-Linked Immuno-Sorbent Assay) inhibition test. The different antibodies are adsorbed on polystyrene plates and a binding curve of albumin labeled with alkaline phosphatase is established for each antibody. The saturation curves for each antibody have a sigmoid appearance, and the amount of labeled albumin corresponding to 50% binding was chosen to study the inhibition by each antibody.

Inhibition was carried out with the sample of recombinant albumin and compared with that obtained with a sample of plasma albumin (Sigma). The pannels A, B, and C of Figure 44 shows that, for the nine antibodies tested, the recombinant albumin is as inhibitory as native albumin. Given the extreme sensitivity of the test, the differences observed are not likely to be significant.

Example 7: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE SECRETION OF INTERLEUKIN-1β.

E.7.1 Synthesis of the IL-1β structural gene

The chemical synthesis of the structural gene corresponding to IL-1β has already been described (Jung, G. et al., Ann. Inst. Pasteur/Microbiol. 139 (1988) 129-146): in brief, the structural gene was assembled from 14 synthetic oligodeoxynucleotides as a NdeI-HindIII restriction fragment. Compared with the sequence published by Auron et al. (Proc. Natl. Acad. Sci. 81 (1984) 7907- 7911), the synthetic gene used in the present example contains the following modifications: i) change of the AGC codon (Ser) at position 483 for the TCC codon in order to eliminate the HindIII site; ii) substitution of a T by a C at position 506 to eliminate the NdeI site; iii) creation of an EcoRI site followed by an NdeI site at position 435 (GAATTCATATG) including the translation initiation methionine (bold letters); iv) addition of the sequence AAGCTT at position 896 in order to introduce a HindIII site immediately downstream from the translation termination codon.

The structural gene encoding the recombinant IL-1β is thus available as a EcoRI-HindIII restriction fragment contained in a derivative of plasmid pUC19 designated pCI131 (Figure 46).

E.7.2 Construction of a secretion vector containing IL-1β under the control of the PGK promoter of S. cerevisiae and the killer toxin secretion signal of K. lactis

A DNA fragment corresponding to the secretion signal ("pre" region) of the toxin gene of killer plasmid k1 of K. lactis (Stark, M.J.R. and Boyd, A., EMBO J. 5 (1986) 1955-2002) was assembled from the following two synthetic oligodeoxynucleotides:

SqA : 5′-AATTATGAATATATTTTACATATTTTTGTTTTTGCTGTCATTCGTTCAAGGTAAAAG-3′

SqB : 5′-AATTCTTTTACCTTGAACGAATGACAGCAAAAACAAAAATATGTAAAATATATTCAT-3′

These two oligodeoxynucleotides were hybridized, thereby reconstituting the signal sequence of the killer toxin followed by a dipeptide LysArg which represents a potential cleavage site recognizable by an endopeptidase produced by the KEX1 gene of K. lactis (Tanguy-Rougeau, C. et al., FEBS Letters 234 - (1988) 464-470). This sequence is flanked by cohesive termini compatible with those of an EcoRI site. In this construction, only the terminus situated downstream from the signal sequence can regenerate a functional EcoRI site. This DNA fragment was cloned in the desired orientation into the single EcoRI site of plasmid pEPGK41 thereby generating plasmid pSPGK1 (Figure 45). pEPGK41 contains a SalI-BamHI cassette containing the promoter and terminator of the PGK gene of S. cerevisiae, identical to that present in plasmid pYG12 (already described in Example 3) with the exception of the HindIII cloning site which has been replaced by an EcoRI site (see the sequence given in Figure 45). As a result of the strategy used, plasmid pSPGK1 possesses a unique EcoRI site permitting the cloning of structural genes downstream from the synthetic signals sequence.

In order to obtain the structural gene encoding IL-1β in the form of an EcoRI restriction fragment, plasmid pCI131 (see section E.7.1) was linearized with the enzyme HindIII, treated with the Klenow fragment of E. coli DNA polymerase I to render the termini blunt-ended and ligated with a synthetic EcoRI linker (5′-GGAATTCC-3′). After cleavage with the enzyme EcoRI, the fragment corresponding to Met-IL-1β was purified by electroelution and cloned into the EcoRI site of plasmid pSPGK1 to generate plasmid pSPGK-IL17 (Figure 46). In this construction, the IL-1β coding sequence is in translational frame with the secretion signal derived from the killer toxin. The beginning of the mature IL-1β gene (AlaProVal...) is separated from the cleavage site recognized by the signal peptidase (GlnGly ^) by the pentapeptide LysArgIleHisMet.

Plasmid pSPGK-IL17 is the source of a SalI-HindIII restriction fragment corresponding to the expression cassette (PGK promoter/secretion signal-IL-1β/PGK terminator); this fragment was purified by electroelution and cloned into plasmid pCXJ1, cleaved by the same enzymes to generate plasmid pSPGK-IL21 (Figure 47). Plasmid pUC4K (Pharmacia) is the source of the gene for resistance to geneticin (G418), which was purified by electroelution as a SalI restriction fragment. This fragment was then cloned into the SalI site of plasmid pSPGK-IL21 to generate the IL-1β expression plasmid pSPGK-IL31 (Figure 48).

E.7.3 Construction of a secretion vector containing IL-1β under the control of the PHO5 promoter of S. cerevisiae.

The importance of the PHO5 promoter lies in the fact that it represents an inducible expression system in K. lactis: as in S. cerevisiae, this promoter is repressed at high inorganic phosphate concentration and induced in medium lacking phosphate (Chen, X.J. and Fukuhara, H., Gene 369 (1988) 181-192).

the EcoRI fragment corresponding to the secretion signal of the toxin gene of K. lactis killer plasmid k1 (see section E.7.2) was cloned in the desired orientation into the EcoRI site of plasmid pEPHO (see section E.3.4) to generate plasmid pSPHO4 (Figure 49). The EcoRI restriction fragment corresponding to Met-IL-1β was purified from plasmid pSPGK-IL17 and cloned into the EcoRI site of plasmid pSPHO4 to generate plasmid pSPHO-IL14 (Figure 50). This plasmid is the source of a SalI-HindIII restriction fragment corresponding to the expression cassette (PHO5 promoter/secretion signal-IL-1β/PHO5 terminator); this fragment was purified by electroelution and cloned into plasmid pCXJ1 cut by the same enzymes to generate plasmid pSPHO-IL23 (Figure 51). The SalI restriction fragment containing the gene for resistance to geneticin (G418) was purified by electroelution from plasmid pUC4K and then cloned into the SalI site of plasmid pSPHO-IL23 to generate the IL-1β expression plasmid pSPHO-IL35 (Figure 52).

E.7.4 Mitotic stability of IL-1β-expressing pKD1 derived plasmids under non-selective growth conditions

The mitotic stability of plasmids pSPGK-IL31 and pSPHO-IL35 was defined as the percentage of cells which have retained the capacity to grow on minimum selective medium (not supplemented with uracil) after growth of a defined number of generations in non-selective medium (YPD). As shown in Figure 53, these 2 plasmids are remarkably stable, although there is a different stability between the construction using the PGK promoter compared with the construction containing the PHO5 promoter: after 40 cell generations in a non-selective medium, 75% (promoter induced) to 93% (promoter not induced) of the cells have maintained plasmid pSPHO-IL35, whereas plasmid pSPGK-IL31 is retained in only 44% of the cells. These results show the importance of an inducible expression system uncoupling the growth phase of a yeast culture from the phase of production of a potentially toxic heterologous protein, from the standpoint of the industrial exploitation of expression vectors derived from plasmid pKD1.

E.7.5 Secretion of IL-1β.

The constructions pSPGK-IL31 and pSPHO-IL35 were transferred into K. lactis strain MW98-8C according to the techniques already described. After selection of the transformants in minimum medium devoid of uracil, a few clones were inoculated in YPD medium containing 200 µg/ml of G418 and their capacity to secrete IL-1β was studied.

Samples of the culture supernatant were treated with ethanol (60 minutes at -20°C at a final concentration of 60%) in order to precipitate the secreted IL-1β. The precipitated material was recovered by centrifugation (20 minutes at 15000 g) and resolubilized in sample buffer by heating to 95°C for 15 minutes in one tenth of the original volume before being deposited on a 12.5% polyacrylamide gel (Laemli, U.K., Nature 227 (1970) 680 - 685). After electrophoresis of the samples, IL-1β secretion was detected either in denaturing gels stained with Coomassie Blue (Figure 54) or by immunological detection after blotting the gel onto nitrocellulose membranes and using polyclonal antibodies directed towards human IL-1β - (Genzyme), with or without prior treatment of the samples with endoglycosidase H according to the directions of the manufacture (Boehringer Mannheim).

The immunological signal obtained from the supernatants of the yeasts transformed with the construction corresponding to IL-1β is sensitive to treatment with endoglycosidase H (decrease in the apparent molecular weight from 24 kD to 19 kD), demonstrating that the recombinant IL-1β of yeast is glycosylated. This result is in agreement with the presence of a potential N-glycosylation site in the N-terminal portion of IL-1β (Asn at position 7).

2 ml of culture supernatant of K. lactis MW98-8C, transformed with plasmid pSPHO-IL35 and grown in an Erlenmeyer in medium lacking inorganic phosphate (Chen, X.J. and Fukuhara, H., Gene 369 (1988) 181-192), were directly applied to a reversed-phase column in order to purify the secreted IL-1β: the elution peak corresponding to 35% of acetonitrile was collected and corresponds to the recombinant interleukin. The quantity of material purified by this technique was determined by spectrophotometry and allows to estimate that IL-1β is secreted by K. lactis at the level of 35 mg per liter of culture supernatant.

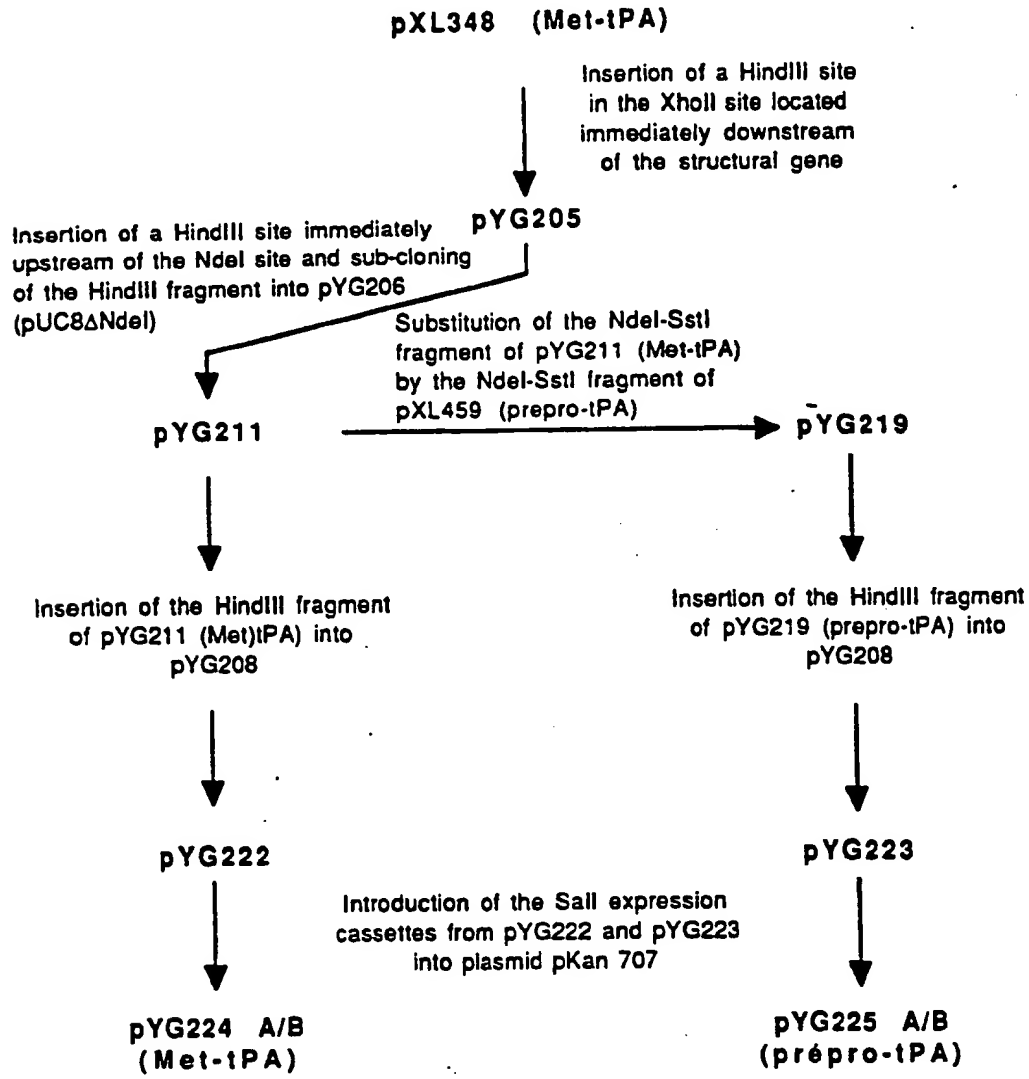
Sequencing of the N-terminal portion of the purified IL-1β (automated Edman degradation, Applied Biosystems) indicates the total absence of the "pre" region of the killer toxin and hence the effective maturation by K. lactis strain MW98-8C at the GlnGly^ cleavage site which would be recognized by a yeast signal peptidase (Stark, M.J.R. and Boyd, A., EMBO J. 5 (1986) 1955-2002). As a result of the strategy used in the construction of the IL-1β-expressing cassette present in plasmid pSPHO-IL35, the N-terminal end of the recombinant IL-1β cannot be identical to that of mature human IL-1β: the arrangement of the first 11 residues determined by microsequencing (LysArgIleHisMetAlaProValArgSerLeu) confirms the presence of several additional amino acids (LysArgIleHisMet) upstream from the sequence corresponding to mature human IL-1β, and demonstrates, in particular, that the potential site of cleavage by the enzyme KEX1 of K. lactis (LysArg^) is not recognized in this sequence context. The creation by site directed mutagenesis of a correct junction between the secretion signal for the killer toxin and the structural gene for mature IL-1β (GlnGly^AlaProVal) should hence enable a correctly maturated, secreted IL-1β to be obtained.

Example 8: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE EXPRESSION AND SECRETION OF tPA

E.8.1 Construction of vectors for Met-tPA expression and prepro-tPA secretion under control of the PGK promoter of S. cerevisiae

The structural gene encoding tPA was isolated from plasmids pXL348 (Met-tPA) and pXL459 (prepro-tPA) (Sarmientos, P. et al., Bio/Technology 7 (1989) 495-501), the restriction maps of which are shown in Figure 55.

Starting from plasmid pXL348 (Met-tPA), plasmids pYG224 A/B (Met-tPA) and pYG225 A/B (prepro-tPA) are derived according to the following diagram:



Plasmid pXL348 is digested by the enzyme XhoII, and the restriction mixture is ligated with a phosphorylated XhoII-HindIII adaptor (synthetic oligodeoxynucleotide 5′-GATCAAGCTT-3′); the mixture is then digested by the enzyme HindIII, and the restriction fragment encoding Met-tPA is cloned into the HindIII site of the vector pUC8 to generate the intermediate plasmid pYG205. This plasmid is cut with the enzyme NdeI and ligated with an equimolar mixture of the phosphorylated synthetic oligodeoxynucleotides 5′-TAAGCTTCA-3′ and 5′-TATGAAGCT-3′ which constitute a NdeI-HindIII adaptor; after in vitro ligation, the mixture is digested by HindIII and the restriction fragment encoding Met-tPA is purified by electroelution and cloned into the HindIII site of the vector pYG206 to generate plasmid pYG211. The vector pYG206 corresponds to plasmid pUC8 in which the unique NdeI site has been destroyed by cleavage with the enzyme NdeI and filling-in of the cohesive termini with the Klenow fragment of E. coli polymerase I before

self-ligation of the vector; plasmid pYG211 hence contains a HindIII fragment encoding Met-tPA in which there is a single NdeI restriction site localized at the ATG initiation codon of Met-tPA. In plasmid pYG211, the nucleotide sequence upstream from the translation initiation codon (bold letters) is as follows: 5'-AAGCTTCATATG...-3'.

Plasmid pYG211 is the source of plasmid pYG219, which contains a HindIII fragment encoding prepro-tPA. This plasmid was constructed by substituting the NdeI-SstI fragment of pYG211 by the NdeI-SSti fragment of plasmid pXL459, which contains the N-terminal portion of prepro-tPA (Figure 55); plasmids pYG211 (Met-tPA) and pYG219 (prepro-tPA) are hence strictly isogenic, except that plasmid pYG211 encodes the mature form of tPA immediately preceded by the initiation codon, whereas, in plasmid pYG219, the mature tPA sequence is preceded by its prepro natural exportation sequence.

The HindIII fragments encoding Met-tPA and prepro-tPA were purified by electroelution from plasmids pYG211 and pYG219, and cloned in the correct orientation into the vector pYG208 (c.f. Figure 19A) cut by the same restriction enzyme, which generates the intermediate plasmids pYG222 and pYG223. These plasmids contain an expression cassette available as a SalI restriction fragment, which was then cloned into the vector pKan707 to generate plasmids pYG224 A/B (Met-tPA) and pYG225 A/B (prepro-tPA). The A and B orientations of the SalI cassettes with respect to the vector were defined as above for plasmids pYG221 A/B (prepro-HSA) (c.f. section E.3.2.).

E.8.2 Secretion of tPA

The construction pYG224 A/B (Met-tPA) and pYG225 A/B (prepro-tPA) were transferred, in particular, into K. lactis strain MW98-8C according to the techniques described in section E.4.1. After selection of the transformed cells in the presence of G418, a few clones were inoculated in selective YPD medium, and their capacity for expressing and secreting tPA was studied according to the following techniques: (i) in Coomassie blue stained SDS polyacrylamide gels; (ii) by immunological detection after blotting of the gel onto nitrocellulose membrane and use of primary antibodies directed towards human tPA (Biopool), with or without prior treatment of the cell fractions with deglycosylating enzymes, endoglycosidase H or glycopeptidase F, which were used according to the directions of the manufacturer (Boehringer Mannheim); (iii) in a fibrin plate assay on indicator plates of the following composition: 1% agarose, 0.1% fibrinogen (KabiVitrum), 0.2 unit/ml thrombin (Sigma), dissolved in PBS buffer.

The results of these experiments demonstrate that no specific immunological signal is detectable in the untreated culture supernatants (construction pYG225B), or in the cell extracts. The detection method cannot be called into question, since the constructions pYG224 A/B (Met-tPA) give a strong specific signal (between 1 and 10 mg/liter of culture) in the cell extracts and more especially in the fraction corresponding to the insoluble proteins.

The use of deglycosylating enzymes appears as a prerequisite for the immunological detection of the prepro-tPA expressed and secreted by K. lactis under the control of the PGK promoter of S. cerevisiae. This observation may be explained by an heterogeneous hyperglycosylation of this protein when expressed in this strain under control of the PGK promoter. In particular, this hyperglycosylation could be responsible for the weak antigenicity of the yeast recombinant tPA expressed from the PGK promoter.

Figure 56 demonstrates the presence of an enzyme activity in the culture supernatants of K. lactis MW98-8C transformed with plasmid pYG225B (prepro-tPA), whereas it is absent from the culture supernatants of strain MW98-8C transformed with plasmids pKan707 (control vector) or pYG224B (Met-tPA). The detection of a secreted tPA activity hence demonstrates that the prepro-sequence of tPA is functional in K. lactis MW98-8C. The test of tPA activity on indicator plates also demonstrates the presence of activity in the intracellular fractions of strain MW98-8C transformed with the construction Met-tPA (result not documented).

E.8.3 Influence of the host strain on tPA secretion

The choice of a host cell is a fundamental factor as regards the levels of tPA secretion by yeasts capable of replicating derivatives of plasmid pKan707. The capacity of yeasts of the genus Kluyveromyces to secrete tPA cloned into the vector pKan707 was tested as above on fibrinogen-agar indicator plates. K. lactis strains ATCC 34609 and ATCC 34610, ATCC 36906 (K. drosophilarum), and K. fragilis ATCC 36534 and ATCC 36907 have a potential for tPA secretion of the same order of magnitude as that of K. lactis strain MW98-8C, whereas tests carried out under the same conditions on strains ATCC 12424 (K. fragilis),

ATCC 24178 (K. wickerhamii) and ATCC 56500 (K. waltii) did not enable tPA activity to be detected in the culture supernatants.

Example 9: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE EXPRESSION AND SECRETION OF TIMP

E.9.1 Construction of vectors for expression and secretion of TIMP under control of the PGK promoter of S. cerevisiae

The complete structural gene encoding TIMP was isolated from plasmid pPV2-TIMP. This vector consists of the TIMP cDNA available as a HindIII restriction fragment (Kaczorek, M. et al., Bio/Technol. 5 - (1987) 595-598). This restriction fragment was purified by electroelution and cloned in the correct orientation into the HindIII site of the construction intermediate pYG208 (c.f. Figure 19A) to generate plasmid pYG220. This plasmid is the source of a SAII fragment corresponding to the expression cassette (P G K promoter/TIMP/PGK terminator) which was cloned into the SalI site of the vector pKan707 (c.f. Figure 13) to generate the expression plasmids pYG226 A/B, which differ from one another only in the orientation of this SalI fragment with respect to the vector. The A and B orientations of plasmids pYG226 A/B were defined as above for plasmids pYG221 A/B (prepro-HSA), pYG224 A/B (Met-tPA) and pYG225 A/B (prepro-tPA).

E.9.2 Secretion of TIMP

The construction pYG226 A/B (TIMP) were transferred into K. lactis strain MW98-8C according to the techniques already described. After selection of the transformed cells in the presence of G418, a few clones were inoculated in selective YPD medium (G418, 200 µg/ml) and their capacity to express and secrete TIMP was studied, either in Coomassie blue stained SDS polyacrylamide gels, or by immunological detection after blotting of the gel onto the nitrocellulose membranes and use of polyclonal antibodies directed towards human TIMP (Rhône-Poulenc Santé), with or without prior treatment of cell fractions with endoglycosidase H.

An immunological signal corresponding to TIMP is detectable in the non-deglycosylated intracellular extracts (insoluble protein fractions), but not in the non-deglycosylated culture supernatants (Figure 57). This immunological signal is sensitive to treatment with deglycosylating enzymes, demonstrating that the yeast recombinant TIMP is N-glycosylated. The secretion of recombinant TIMP is demonstrated after in vitro deglycosylation of a culture supernatant of strain MW98-8C transformed with plasmid pYG226B (Figure 57), indicating that the natural exportation sequence of TIMP is functional in K. lactis MW98-8C.

Note

A sample of strain MW98-8C has been deposited at the Centraalbureau voor Schimmelkulturen (CBS) at Baarn in the Netherlands according to the provisions of the Treaty of Budapest, where it was registered on 16.09.1988 under number CBS 579.88.

Claims

1. A method for the microbiological preparation of human serum albumin (HSA) or one of its variants, wherein a yeast capable of providing for the stable maintenance of an expression cassette containing at least one selectable marker for the transformed yeasts, the DNA of the structural gene for HSA or for said variant under the control of sequences permitting its expression in yeast and, where appropriate, excretion of the protein encoded by this gene into the growth medium, is grown.

2. The method as claimed in claim 1, wherein the expression cassette is integrated in the yeast genome.

3. The process as claimed in claim 1, wherein the expression cassette forms part of a plasmid containing a replication system functioning in yeast and providing for the stable maintenance of said

cassette in this yeast.

4. The method as claimed in one of claims 1, 2 or 3, wherein the yeasts are chosen from the genera Saccharomyces and Kluyveromyces.

5. The method as claimed in one of claims 1, 2 or 3, wherein the yeast is chosen from the genus Kluyveromyces.

6. The method as claimed in one of claims 1,2 or 3, wherein the yeast is chosen from all varieties of Kluyveromyces marxianus.

7. The method as claimed in one of claims 1, 2 or 3, wherein the yeast is Kluyveromyces marxianus var. lactis.

8. The method as claimed in one of claims 3 to 7, wherein the replication system functioning in yeast is all or part of plasmid pKD1 originally isolated from Kluyveromyces marxianus var. drosophilarum, or all or part of the 2 μ plasmid isolated from Saccharomyces cerevisiae, or a combination of elements derived from plasmid pKD1 and from the 2 μ plasmid.

9. The method as claimed in one of claims 3, 6, 7 or 8, wherein the replication system functioning in yeast is all or part of the pKD1 sequence.

10. A method for the preparation of a specified protein, in which method a yeast of the genus Kluyveromyces transformed with an expression plasmid comprising:
- genes A, B and C of plasmid pKD1,
- the inverted repeats of plasmid pKD1,
- the stability locus of plasmid pKD1,
- the origin of replication of pKD1 and an expression cassette containing a DNA encoding the structural gene for said protein under the control of sequences permitting its expression in said yeast,
- a selectable marker for the transformed yeast,
- and, optionally, an origin of replication and a selectable marker for Escherichia coli,

is cultured in a growth medium.

11. The method as claimed in one of claims 8 to 10, wherein the expression cassette and the selectable marker are inserted in the EcoRI site of pKD1.

12. The method as claimed in one of claims 8 to 11, wherein the expression cassette and the selectable marker are inserted in a region of 197 nucleotides defined by the SacI and MstII sites of pKD1, or the SphI site of pKD1.

13. The method as claimed in one of claims 1 to 12, wherein the sequences permitting the expression of the structural gene are chosen from the promoters derived from genes of yeasts of the genus Saccharomyces or Kluyveromyces.

14. The method as claimed in claim 13, wherein these promoters are derived from the glycolytic genes of yeasts of the genus Saccharomyces or Kluyveromyces.

15. The method as claimed in claim 13, wherein the sequences permitting the expression of the structural gene are chosen from the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), enolases (ENO), alcohol dehydrogenases (ADH), lactase (LAC4) or acid phosphatase (PHO5).

16. The method as claimed in claim 13, wherein the sequences permitting the expression of the structural gene are chosen from the genes encoding phosphoglycerate kinase (PGK) or acid phosphatase (PHO5).

17. The method as claimed in one of claims 1 to 16, wherein the sequences encoding said protein are preceded by a protein-exportation sequence which is chosen from the natural N-terminal leader of the said protein.

18. The method as claimed in one of claims 1 to 17, wherein the sequence encoding said protein is preceded by an exportation sequence different from the natural sequence.

19. The method as claimed in claim 18, wherein the sequence encoding the exportation signal of said protein is the sequence obtained from yeast genes encoding the alpha pheromone or killer toxin.

20. The method as claimed in one of claims 1 to 19, wherein the protein is chosen from:
- interleukins
- albumins
- tPA
- TIMP.

21. The method as claimed in claim 20 wherein the sequence encoding the specified protein is chosen from the sequences encoding:
- IL-1β

- prepro-HSA
- Met-HSA
- prepro-tPA
- Met-tPA and
- TIMP.

22. The method as claimed in one of claims 1 to 19, wherein the sequence permitting the excretion of HSA is the prepro natural terminal leader of albumin.

23. The method as claimed in one of claims 1 to 22, wherein the selectable markers for the transformed yeasts are chosen from the genes providing resistance to antibiotics or to copper ions.

24. The method as claimed in claim 23, wherein the selectable sequence is a gene providing resistance to G418.

25. The method as claimed in claim 24, wherein the selectable sequence is a fusion between the ORF1 promoter of the linear plasmid k1 of K. lactis and the aminoglycoside phosphotransferase of the transposon Tn903.

26. The method as claimed in one of claims 1 to 25, wherein the selectable marker is a gene complementing auxotrophies.

27. The method as claimed in claim 1, wherein the strain transformed with a plasmid which contains plasmid pKD1, prepro-HSA DNA, the transcription initiation and termination sequences of the gene encoding phosphoglycerate kinase and the sequences of a fusion between the ORF1 promoter of the linear plasmid k1 of K. marxianus var lactis and the 3'-aminoglycoside phosphotransferase gene of Tn903.

28. The method as claimed in claim 1, wherein the strain is transformed with a plasmid which contains plasmid pKD1, Met-HSA DNA, transcription initiation and termination sequences of the gene encoding phosphoglycerate kinase and the sequences of a fusion between the ORF1 promoter of the linear plasmid k1 of K. marxianus var. lactis and the 3'-aminoglycoside phosphotransferase gene of Tn903.

29. The method as claimed in one of claims 1 to 28, wherein the strain is of the species K. marxianus, K. wickerhamii, K. waltii.

30. The method as claimed in claim 29, wherein the strain is of the species K. bulgaricus, K. marxianus var. drosophilarum, K. marxianus var. marxianus or K. marxianus var. lactis.

31. The method according to anyone of claims 1 to 30, wherein yeasts are cultured in glucose containing medium to which lactose is added between mid-log and early stationary growth phase.

32. Human serum albumin, when obtained by the method as claimed in one of claims 1 to 31.

33. The application of human serum albumin as claimed in claim 32, by way of a medicinal product.

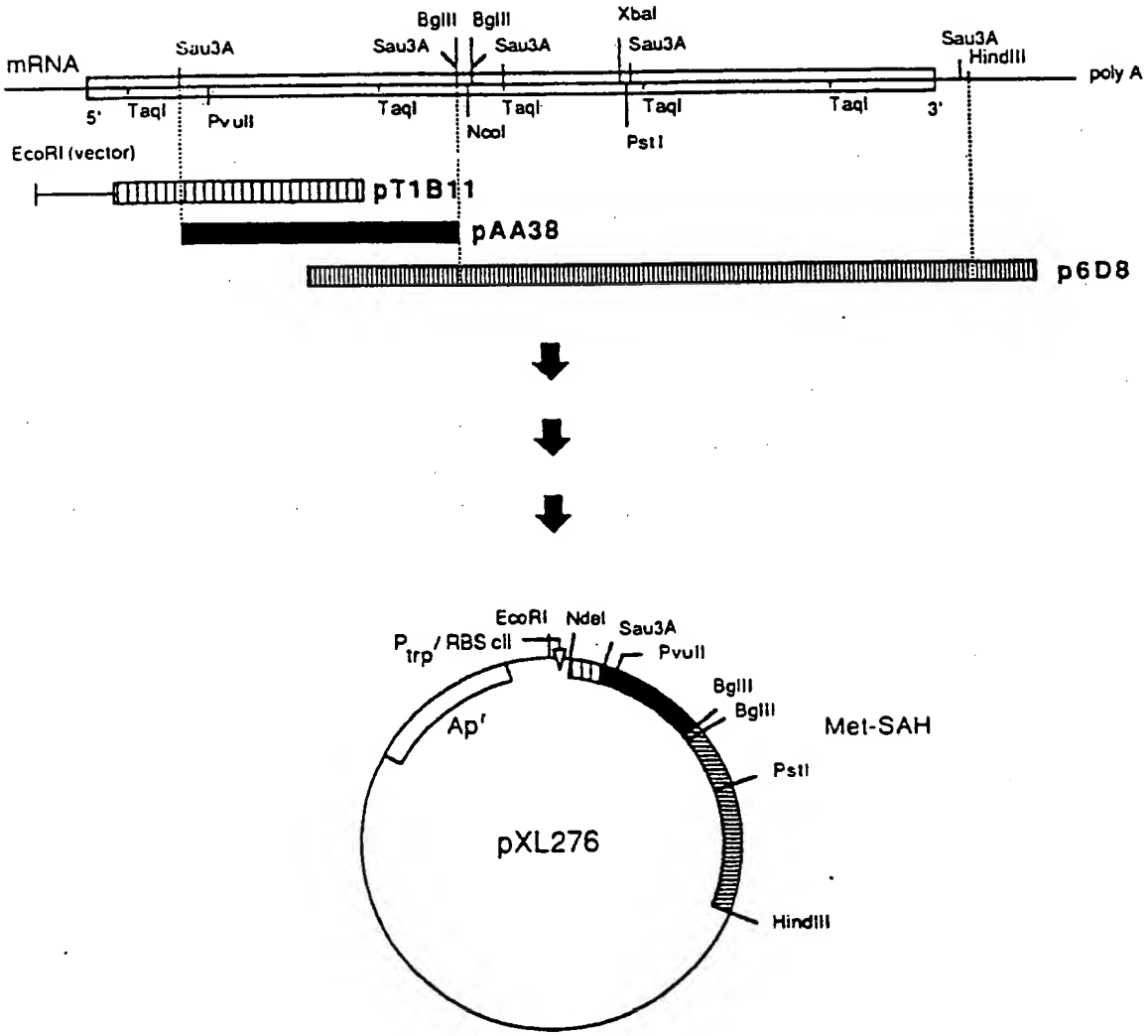

mRNA
Sau3A
BglII
BglII
Sau3A
Sau3A
XbaI
Sau3A
Sau3A
HindIII
poly A
5'
TaqI
PvuII
TaqI
TaqI
NcoI
TaqI
PstI
TaqI
3'
EcoRI (vector)
pT1B11
pAA38
p6D8
EcoRI
NdeI
Sau3A
PvuII
BglII
BglII
Met-SAH
PstI
HindIII
Ptrp / RBS cII
Ap'
pXL276

FIG_1

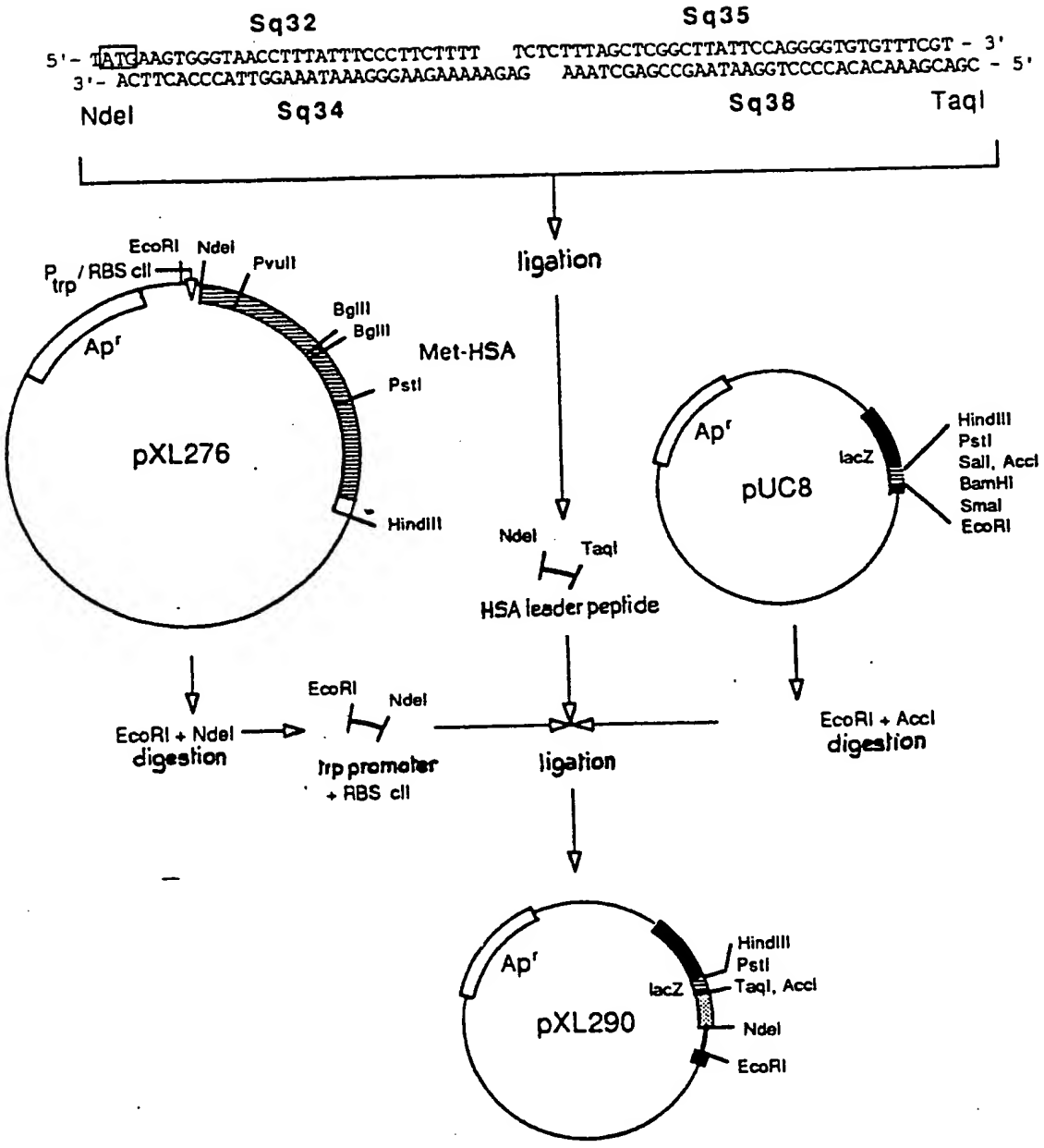

Sq32
Sq35
5'- TATGAAGTGGGTAACCTTTATTTCCCTTCTTTT TCTCTTTAGCTCGGCTTATTCCAGGGGTGTGTTTCGT - 3'
3'- ACTTCACCCATTGGAAATAAAGGGAAGAAAAAGAG AAATCGAGCCGAATAAGGTCCCCACACAAAGCAGC - 5'
NdeI
Sq34
Sq38
TaqI
ligation
EcoRI
NdeI
PvuII
Ptrp / RBS cII
BglII
BglII
Met-HSA
PstI
Apr
pXL276
HindIII
NdeI
TaqI
HSA leader peptide
Apr
lacZ
pUC8
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
EcoRI + NdeI
digestion
EcoRI
NdeI
trp promoter
+ RBS cII
ligation
EcoRI + AccI
digestion
Apr
pXL290
HindIII
PstI
lacZ
TaqI, AccI
NdeI
EcoRI

FIG. 2

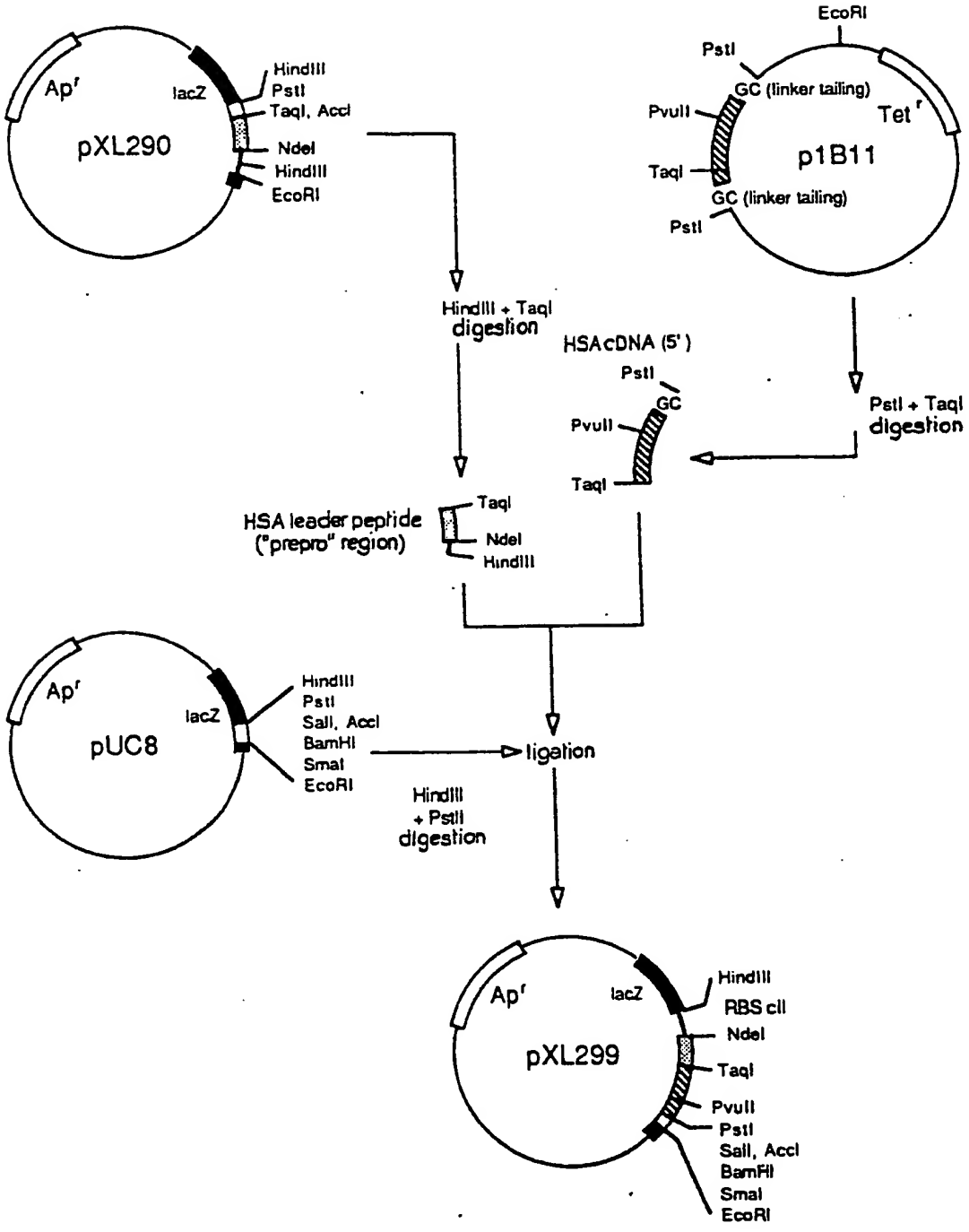

Ap'
lacZ
pXL290
HindIII
PstI
TaqI, AccI
NdeI
HindIII
EcoRI
HindIII + TaqI
digestion
EcoRI
PstI
GC (linker tailing)
PvuII
Tet'
p1B11
TaqI
GC (linker tailing)
PstI
HSAcDNA (5')
PstI
GC
PvuII
TaqI
PstI + TaqI
digestion
HSA leader peptide
("prepro" region)
TaqI
NdeI
HindIII
Ap'
lacZ
pUC8
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
ligation
HindIII
+ PstI
digestion
Ap'
lacZ
pXL299
HindIII
RBS cII
NdeI
TaqI
PvuII
PstI
SalI, AccI
BamHI
SmaI
EcoRI

FIG_3

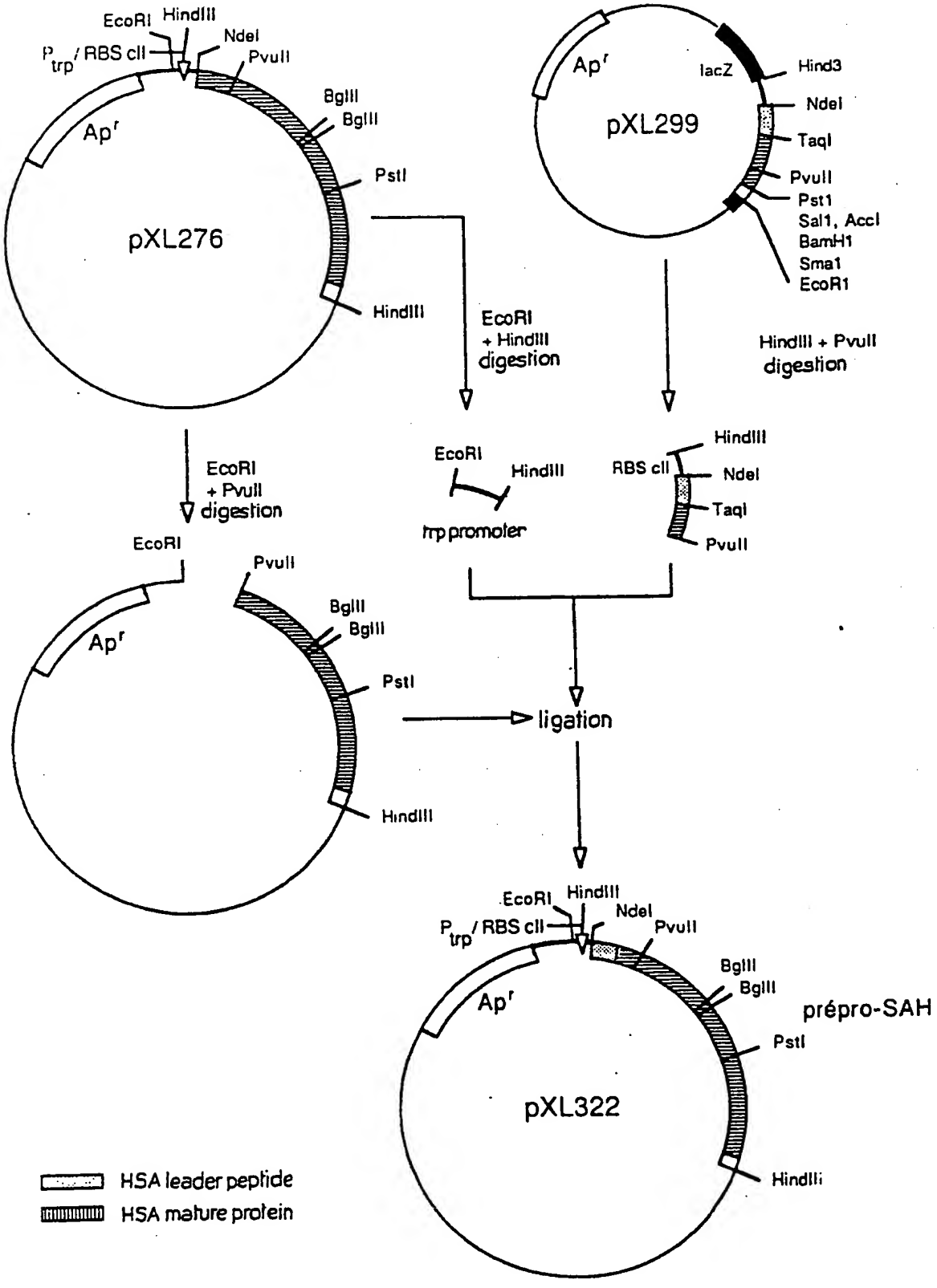

EcoRI
HindIII
NdeI
P_{trp} / RBS cII
PvuII
BglII
BglII
PstI
Ap^r
pXL276
HindIII
EcoRI
+ HindIII
digestion
Ap^r
lacZ
Hind3
pXL299
NdeI
TaqI
PvuII
Pst1
Sal1, AccI
BamH1
Sma1
EcoR1
HindIII + PvuII
digestion
EcoRI
+ PvuII
digestion
EcoRI
HindIII
trp promoter
RBS cII
HindIII
NdeI
TaqI
PvuII
EcoRI
PvuII
BglII
BglII
Ap^r
PstI
HindIII
ligation
EcoRI
HindIII
NdeI
P_{trp} / RBS cII
PvuII
BglII
BglII
Ap^r
PstI
pXL322
HindIII
prépro-SAH
HSA leader peptide
HSA mature protein

FIG_4

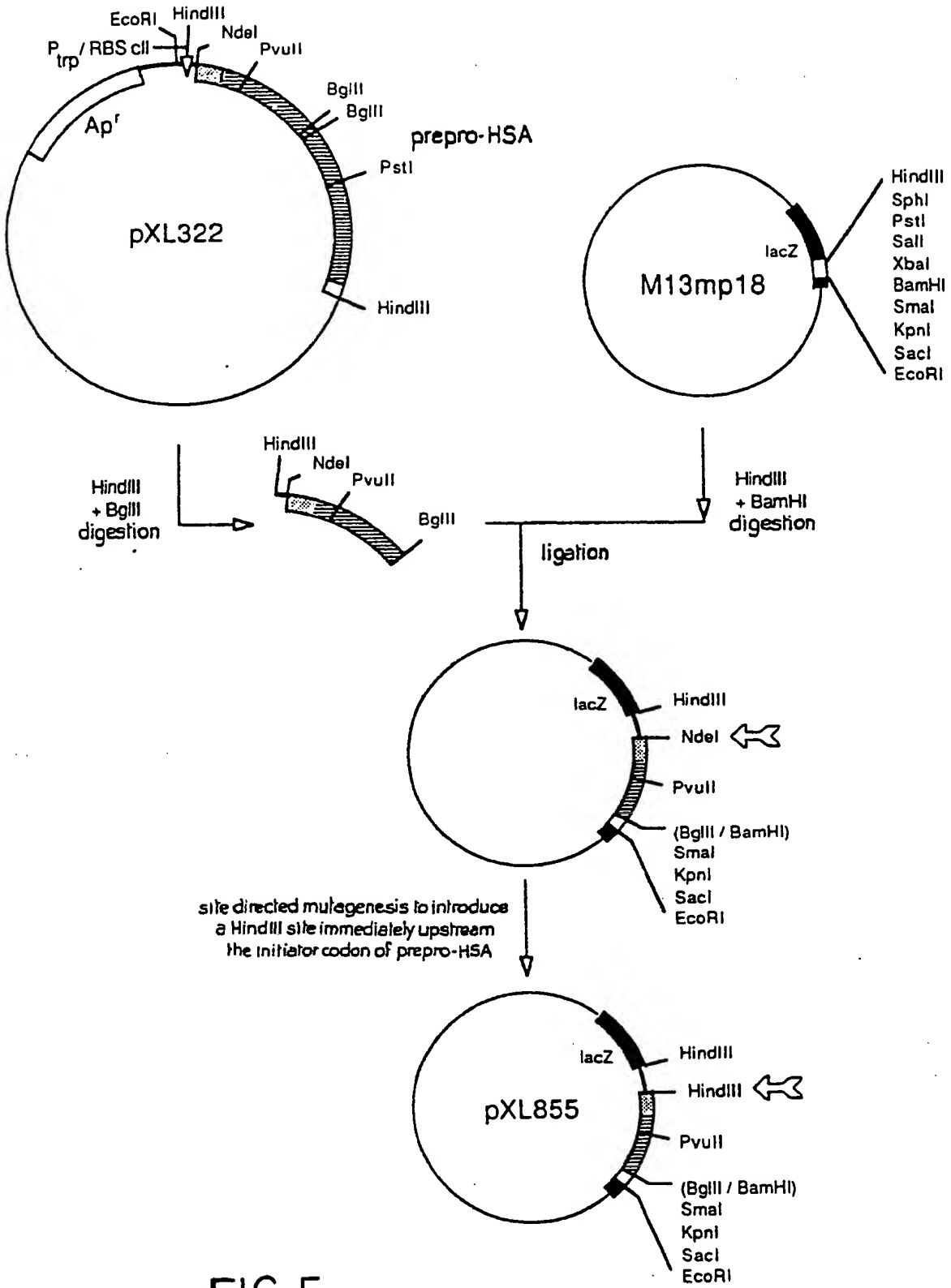

EcoRI
HindIII
NdeI
PvuII
P_{trp} / RBS cII
BglII
BglII
prepro-HSA
Ap^r
PstI
pXL322
HindIII
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
lacZ
M13mp18
HindIII
+ BglII
digestion
HindIII
NdeI
PvuII
BglII
HindIII
+ BamHI
digestion
ligation
lacZ
HindIII
NdeI
PvuII
(BglII / BamHI)
SmaI
KpnI
SacI
EcoRI
site directed mutagenesis to introduce
a HindIII site immediately upstream
the initiator codon of prepro-HSA
lacZ
HindIII
HindIII
pXL855
PvuII
(BglII / BamHI)
SmaI
KpnI
SacI
EcoRI

FIG_5

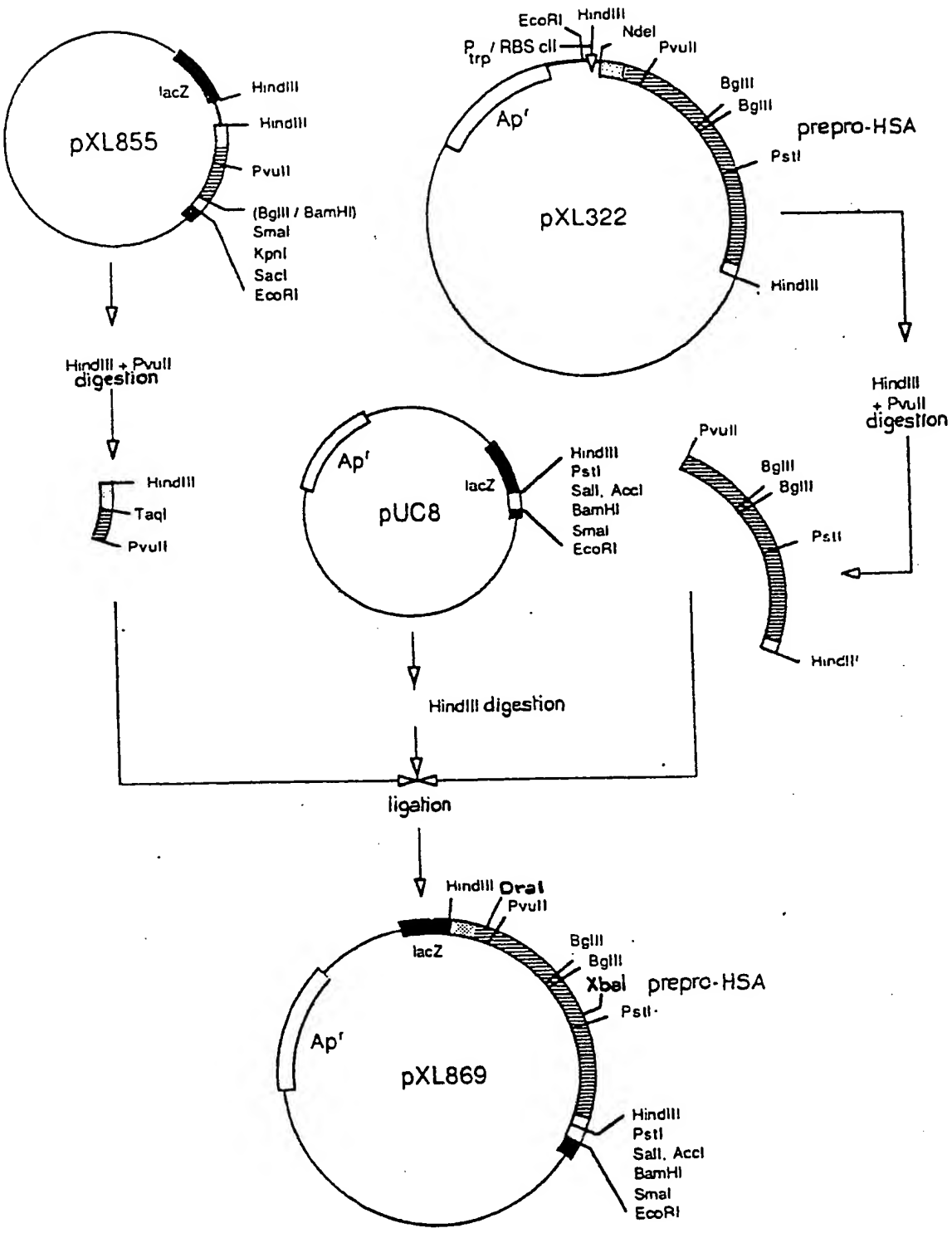

pXL855
lacZ
HindIII
HindIII
PvuII
(BglII / BamHI)
SmaI
KpnI
SacI
EcoRI
HindIII + PvuII digestion
HindIII
TaqI
PvuII
pXL322
EcoRI
HindIII
NdeI
PvuII
P trp / RBS cII
Ap r
BglII
BglII
prepro-HSA
PstI
HindIII
HindIII + PvuII digestion
PvuII
BglII
BglII
PstI
HindIII
pUC8
Ap r
lacZ
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
HindIII digestion
ligation
HindIII OraI
PvuII
lacZ
BglII
BglII
XbaI
prepro-HSA
PstI
Ap r
pXL869
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI

FIG.6

```
        MetLysTrpValThrPheIleSerLeuLeuPheLeuPheSerSerAlaTyrSerArgGlyValPheArg
  AAGCTTATGAAGTGGGTAACCTTTATTTCCCTTCTTTTTCTCTTTAGCTCGGCTTATTCCAGGGGTGTGTTTCGT
  1         11         21         31         41         51         61         71

  ArgAspAlaHisLysSerGluValAlaHisArgPheLysAspLeuGlyGluGluAsnPheLysAlaLeuValLeu
  CGAGATGCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAAAGCCTTGGTGTTG
 76         86         96        106        116        126        136        146

  IleAlaPheAlaGlnTyrLeuGlnGlnCysProPheGluAspHisValLysLeuValAsnGluValThrGluPhe
  ATTGCCTTTGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCATGTAAAATTAGTGAATGAAGTAACTGAATTT
151        161        171        181        191        201        211        221

  AlaLysThrCysValAlaAspGluSerAlaGluAsnCysAspLysSerLeuHisThrLeuPheGlyAspLysLeu
  GCAAAAACATGTGTTGCTGATGAGTCAGCTGAAAATTGTGACAAATCACTTCATACCCTTTTTGGAGACAAATTA
226        236        246        256        266        276        286        296

  CysThrValAlaThrLeuArgGluThrTyrGlyGluMetAlaAspCysCysAlaLysGlnGluProGluArgAsn
  TGCACAGTTGCAACTCTTCGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCTGAGAGAAAT
301        311        321        331        341        351        361        371

  GluCysPheLeuGlnHisLysAspAspAsnProAsnLeuProArgLeuValArgProGluValAspValMetCys
  GAATGCTTCTTGCAACACAAAGATGACAATCCAAATCTCCCCCGATTGGTGAGACCAGAGGTTGATGTGATGTGC
376        386        396        406        416        426        436        446

  ThrAlaPheHisAspAsnGluGluThrPheLeuLysLysTyrLeuTyrGluIleAlaArgArgHisProTyrPhe
  ACTGCTTTTCATGACAATGAAGAGACATTTTTGAAAAAATACTTATATGAAATTGCCAGAAGACATCCTTACTTT
451        461        471        481        491        501        511        521

  TyrAlaProGluLeuLeuPhePheAlaLysArgTyrLysAlaAlaPheThrGluCysCysGlnAlaAlaAspLys
  TATGCCCCGGAACTCCTTTTCTTTGCTAAAAGGTATAAAGCTGCTTTTACAGAATGTTGCCAAGCTGCTGATAAA
526        536        546        556        566        576        586        596

  AlaAlaCysLeuLeuProLysLeuAspGluLeuArgAspGluGlyLysAlaSerSerAlaLysGlnArgLeuLys
  GCAGCCTGCCTGTTGCCAAAGCTCGATGAACTTCGGGATGAAGGGAAGGCTTCGTCTGCCAAACAGAGACTCAAG
601        611        621        631        641        651        661        671

  CysAlaSerLeuGlnLysPheGlyGluArgAlaPheLysAlaTrpAlaValAlaArgLeuSerGlnArgPhePro
  TGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAAAGCATGGGCAGTAGCTCGCCTGAGCCAGAGATTTCCC
676        686        696        706        716        726        736        746

  LysAlaGluPheAlaGluValSerLysLeuValThrAspLeuThrLysValHisThrGluCysCysHisGlyAsp
  AAAGCTGAGTTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCAAAGTCCACACGGAATGCTGCCATGGAGAT
751        761        771        781        791        801        811        821

  LeuLeuGluCysAlaAspAspArgAlaAspLeuAlaLysTyrIleCysGluAsnGlnAspSerIleSerSerLys
  CTGCTTGAATGTGCTGATGACAGGGCGGACCTTGCCAAGTATATCTGTGAAAATCAAGATTCGATCTCCAGTAAA
826        836        846        856        866        876        886        896

  LeuLysGluCysCysGluLysProLeuLeuGluLysSerHisCysIleAlaGluValGluAsnAspGluMetPro
  CTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCATTGCCGAAGTGGAAAATGATGAGATGCCT
901        911        921        931        941        951        961        971

  AlaAspLeuProSerLeuAlaAlaAspPheValGluSerLysAspValCysLysAsnTyrAlaGluAlaLysAsp
  GCTGACTTGCCTTCATTAGCGGCTGATTTTGTTGAAAGTAAGGATGTTTGCAAAAACTATGCTGAGGCAAAGGAT
976        986        996       1006       1016       1026       1036       1046

  ValPheLeuGlyMetPheLeuTyrGluTyrAlaArgArgHisProAspTyrSerValValLeuLeuLeuArgLeu
  GTCTTCTTGGGCATGTTTTTGTATGAATATGCAAGAAGGCATCCTGATTACTCTGTCGTACTGCTGCTGAGACTT
1051       1061       1071       1081       1091       1101       1111       1121
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FIG_7

```
   AlaLysThrTyrGluThrThrLeuGluLysCysCysAlaAlaAlaAspProHisGluCysTyrAlaLysValPhe
   GCCAAGACATATGAAACCACTCTAGAGAAGTGCTGTGCCGCTGCAGATCCTCATGAATGCTATGCCAAAGTGTTT
1136       1146       1156       1166       1176       1186       1196

   AspGluPheLysProLeuValGluGluProGlnAsnLeuIleLysGlnAsnCysGluLeuPheGluGlnLeuGly
   GATGAATTTAAACCTCTTGTGGAAGAGCCTCAGAATTTAATCAAACAAAATTGTGAGCTTTTTGAGCAGCTTGGA
1211       1221       1231       1241       1251       1261       1271

   GluTyrLysPheGlnAsnAlaLeuLeuValArgTyrThrLysLysValProGlnValSerThrProThrLeuVal
   GAGTACAAATTCCAGAATGCGCTATTAGTTCGTTACACCAAGAAAGTACCCCAAGTGTCAACTCCAACTCTTGTA
1286       1296       1306       1316       1326       1336       1346

   GluValSerArgAsnLeuGlyLysValGlySerLysCysCysAsnProGluAlaLysArgMetProCysAlaGlu
   GAGGTCTCAAGAAACCTAGGAAAAGTGGGCAGCAAATGTTGTAATCCTGAAGCAAAAAGAATGCCCTGTGCAGAA
1351       1361       1371       1381       1391       1401       1411       1421

   AspTyrLeuSerValValLeuAsnGlnLeuCysValLeuHisGluLysThrProValSerAspArgValThrLys
   GACTATCTATCCGTGGTCCTGAACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACCAAA
1426       1436       1446       1456       1466       1476       1486       1496

   CysCysThrGluSerLeuValAsnArgArgProCysPheSerAlaLeuGluValAspGluThrTyrValProLys
   TGCTGCACAGAATCCTTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCGATGAAACATACGTTCCCAAA
1501       1511       1521       1531       1541       1551       1561       1571

   GluPheAsnAlaGluThrPheThrPheHisAlaAspIleCysThrLeuSerGluLysGluArgGlnIleLysLys
   GAGTTTAATGCTGAAACATTCACCTTCCATGCAGATATATGCACACTTTCTGAGAAGGAGAGACAAATCAAGAAA
1576       1586       1596       1606       1616       1626       1636       1646

   GlnThrAlaLeuValGluLeuValLysHisLysProLysAlaThrLysGluGlnLeuLysAlaValMetAspAsp
   CAAACTGCACTTGTTGAGCTTGTGAAACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGAT
1651       1661       1671       1681       1691       1701       1711       1721

   PheAlaAlaPheValGluLysCysCysLysAlaAspAspLysGluThrCysPheAlaGluGluGlyLysLysLeu
   TTCGCAGCTTTTGTAGAGAAGTGCTGCAAGGCTGACGATAAGGAAACCTGCTTTGCCGAGGAGGGTAAAAAACTT
1726       1736       1746       1756       1766       1776       1786       1796

   ValAlaAlaSerGlnAlaAlaLeuGlyLeu***
   GTTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACATCACATTTAAAAGCATCTCAGCCTACCATGAGAATAA AG
1801       1811       1821       1831       1841       1851       1861       1871

   AAAGAAAATGAAGATCAAAAGCTT
1876       1886       1896
```

FIG_7

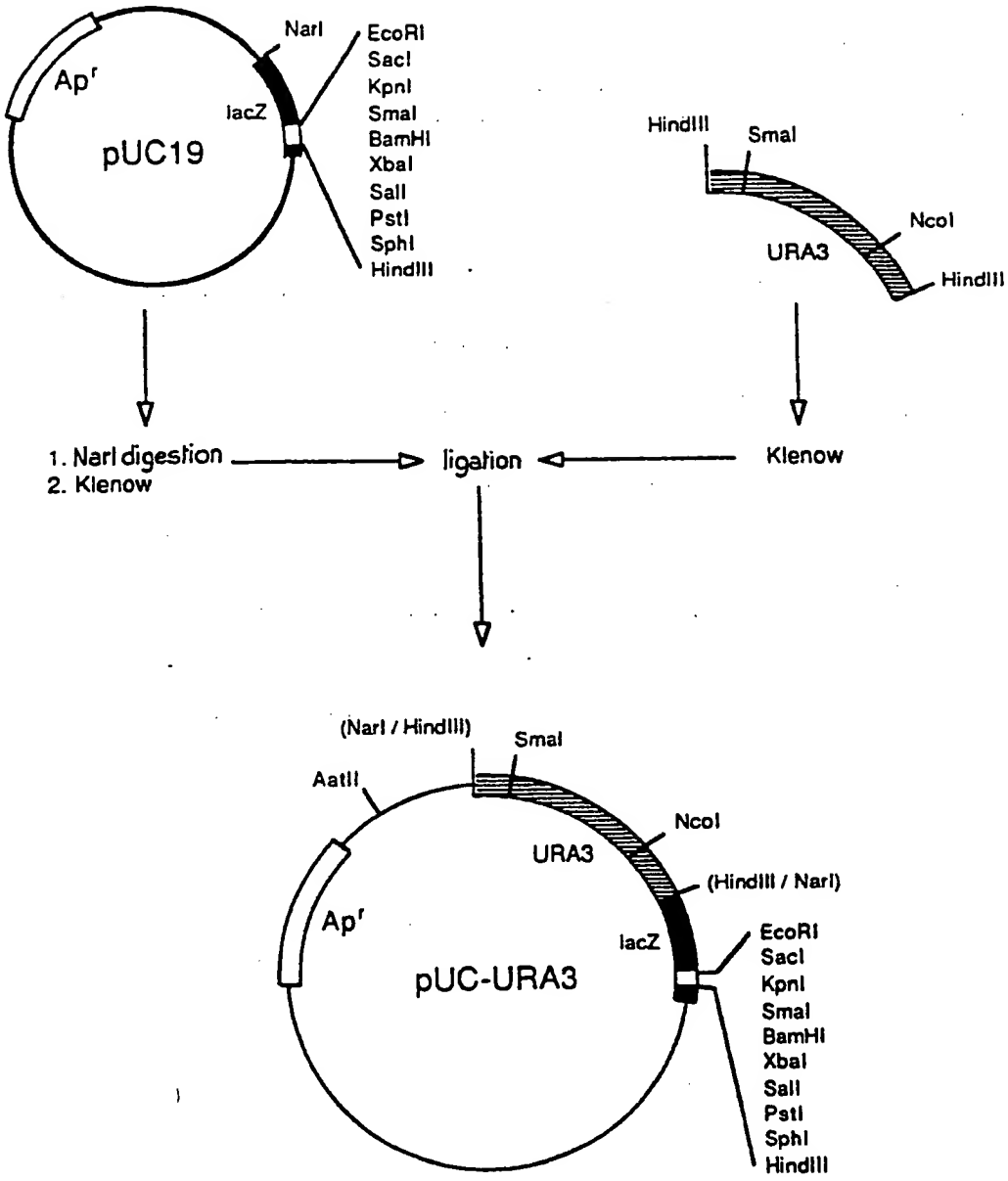

Ap^r
pUC19
lacZ
NarI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
HindIII
SmaI
NcoI
URA3
HindIII
1. NarI digestion
2. Klenow
ligation
Klenow
(NarI / HindIII)
SmaI
AatII
NcoI
URA3
(HindIII / NarI)
Ap^r
lacZ
pUC-URA3
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII

FIG.8

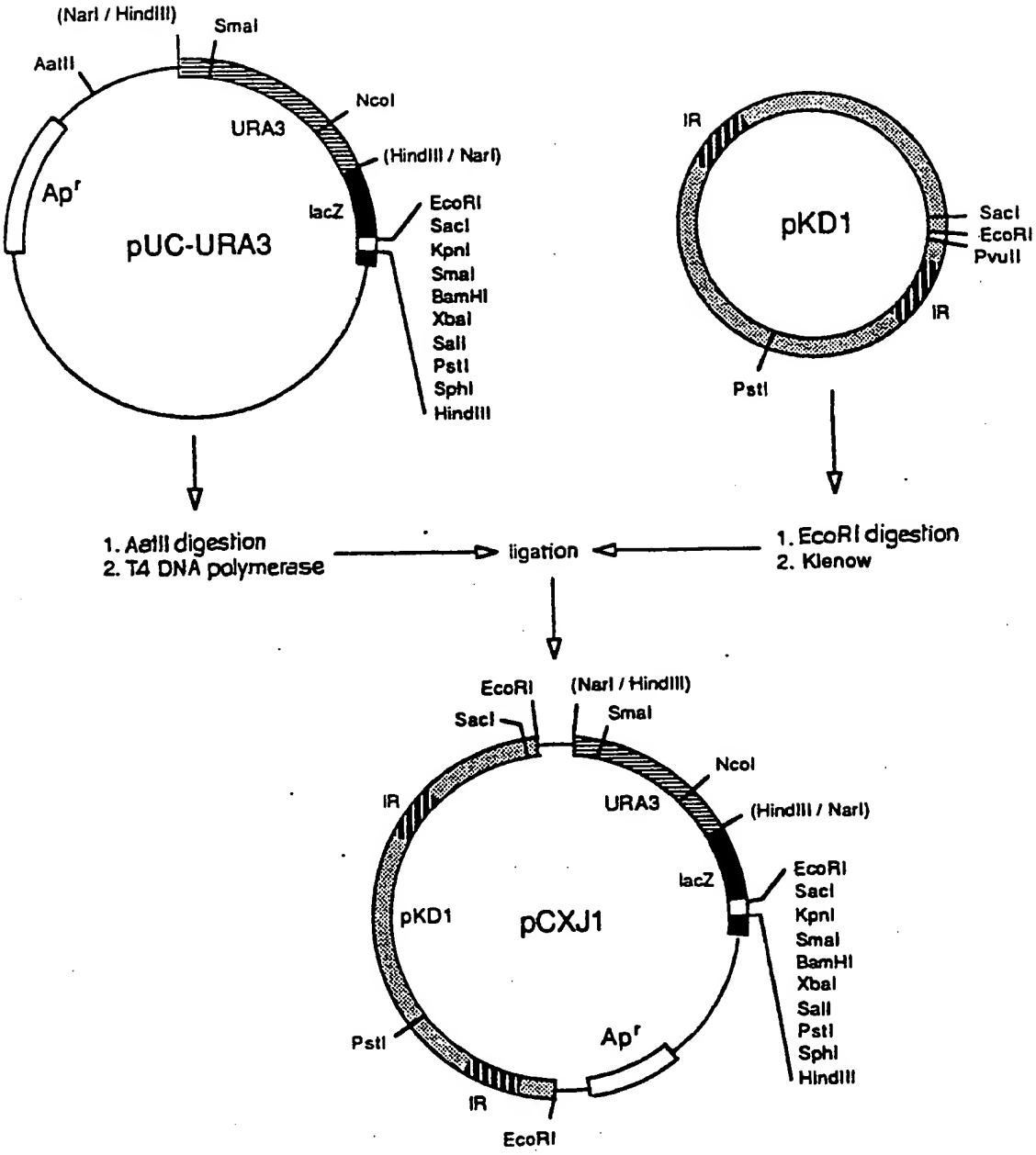

(NarI / HindIII)
SmaI
AatII
NcoI
URA3
(HindIII / NarI)
Ap^r
lacZ
pUC-URA3
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
IR
pKD1
SacI
EcoRI
PvuII
IR
PstI
1. AatII digestion
2. T4 DNA polymerase
ligation
1. EcoRI digestion
2. Klenow
EcoRI
SacI
(NarI / HindIII)
SmaI
NcoI
IR
URA3
(HindIII / NarI)
lacZ
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
pKD1
pCXJ1
Ap^r
PstI
IR
EcoRI

FIG_9

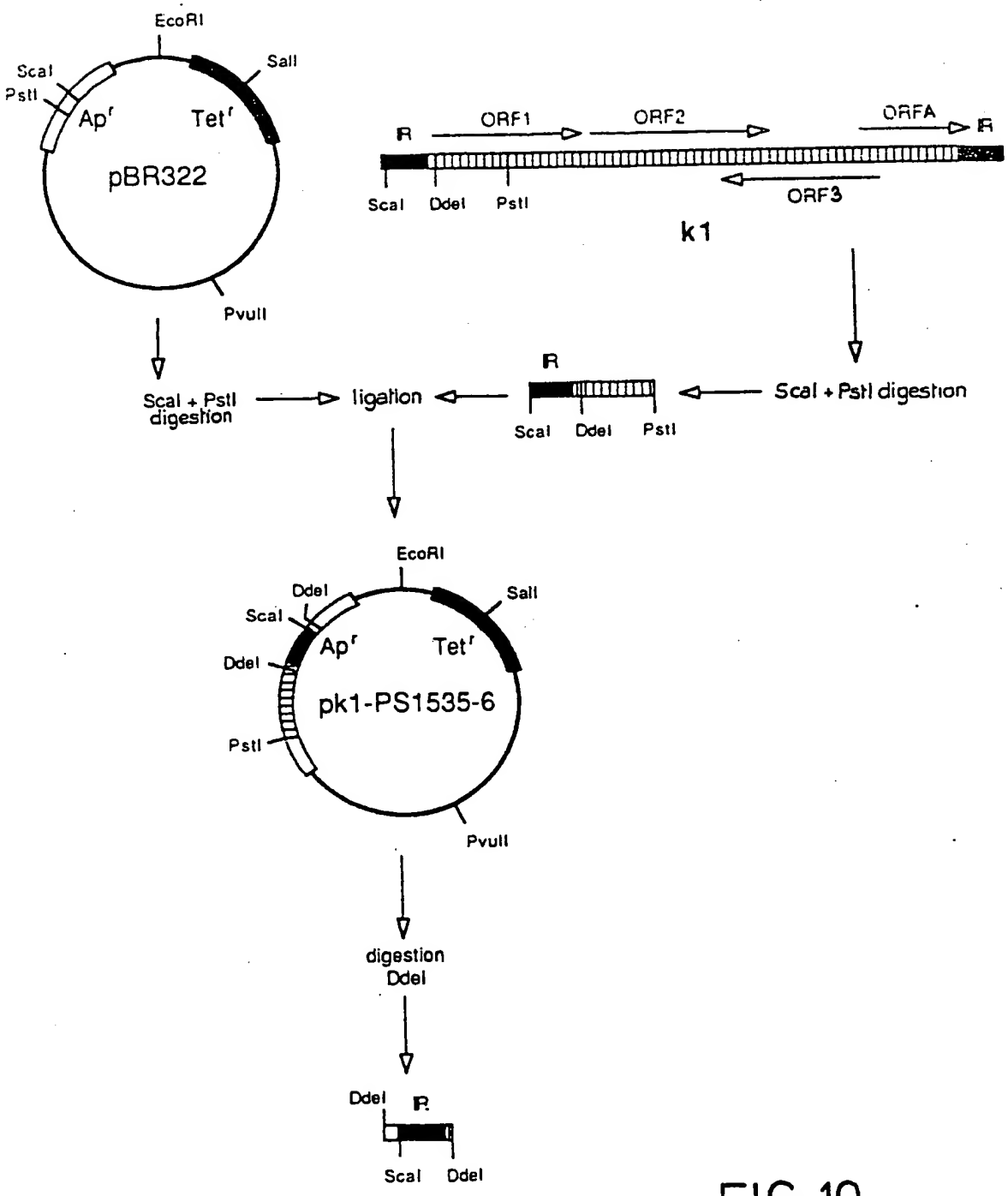

EcoRI
SalI
ScaI
PstI
Ap^r
Tet^r
pBR322
PvuII
IR
ORF1
ORF2
ORFA
ORF3
ScaI
DdeI
PstI
k1
ScaI + PstI digestion
ligation
ScaI + PstI digestion
pk1-PS1535-6
digestion DdeI

FIG_10

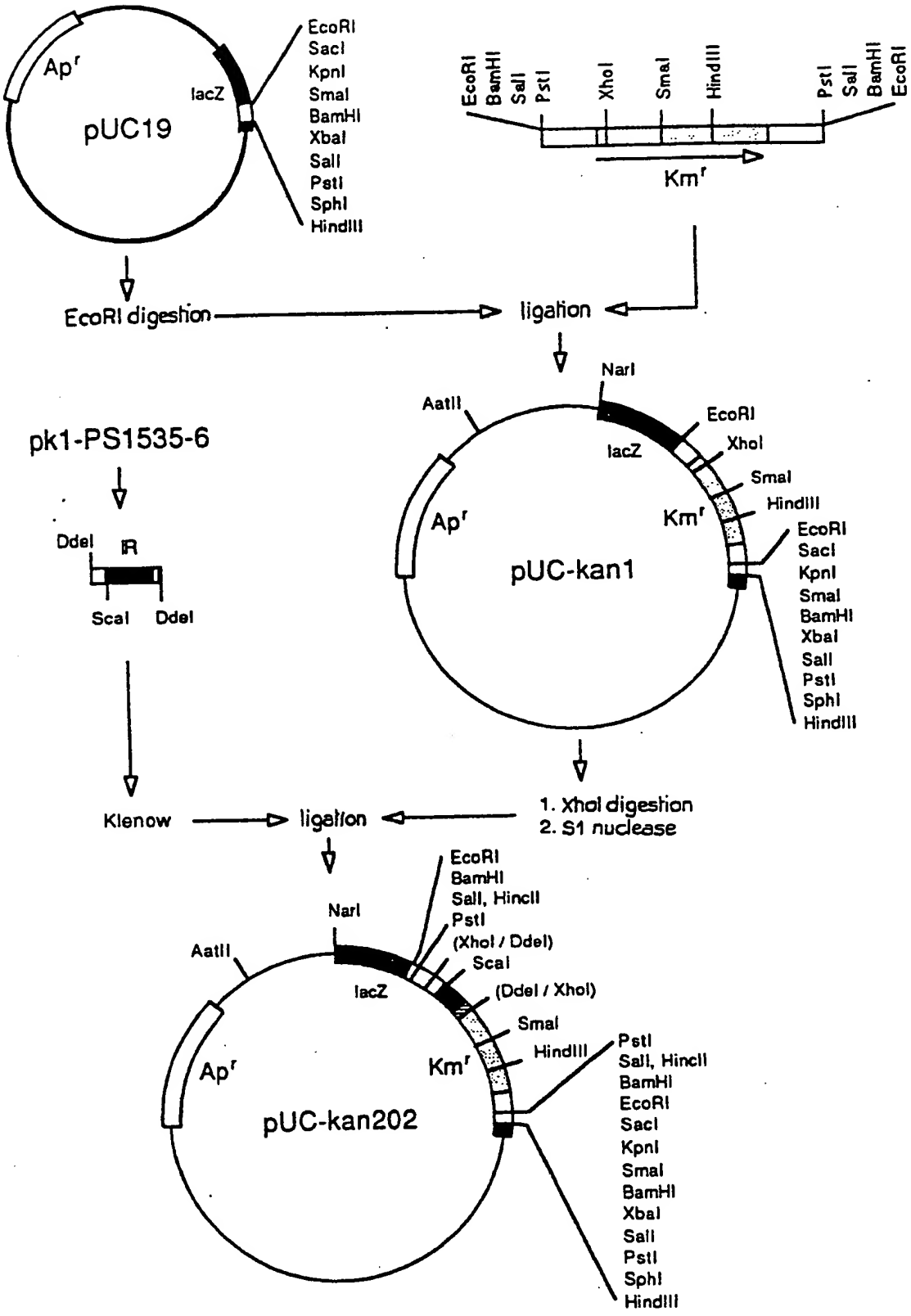

Ap^r
pUC19
lacZ
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
EcoRI BamHI SalI PstI XhoI SmaI HindIII PstI SalI BamHI EcoRI
Km^r
EcoRI digestion
ligation
pk1-PS1535-6
DdeI IR
ScaI DdeI
NarI
AatII
EcoRI
XhoI
SmaI
HindIII
lacZ
Ap^r
Km^r
pUC-kan1
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
1. XhoI digestion
2. S1 nuclease
Klenow
ligation
EcoRI
BamHI
SalI, HincII
PstI
(XhoI / DdeI)
ScaI
(DdeI / XhoI)
SmaI
HindIII
NarI
AatII
lacZ
Ap^r
Km^r
pUC-kan202
PstI
SalI, HincII
BamHI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII

FIG_11

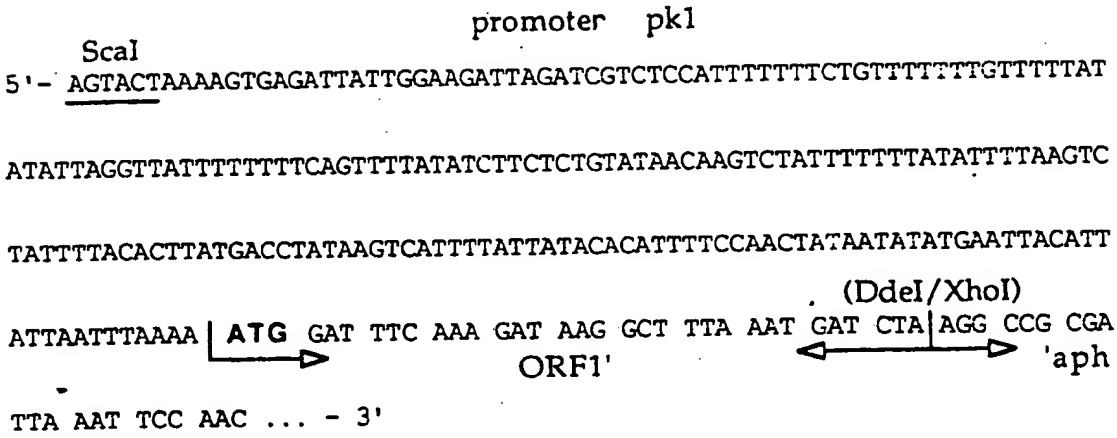

promoter pk1
ScaI
5'- AGTACTAAAAGTGAGATTATTGGAAGATTAGATCGTCTCCATTTTTTTCTGTTTTTTTGTTTTTAT
ATATTAGGTTATTTTTTTTCAGTTTTATATCTTCTCTGTATAACAAGTCTATTTTTTTATATTTTAAGTC
TATTTTACACTTATGACCTATAAGTCATTTTATTATACACATTTTCCAACTATAATATATGAATTACATT
(DdeI/XhoI)
ATTAATTTAAAA ATG GAT TTC AAA GAT AAG GCT TTA AAT GAT CTA AGG CCG CGA
ORF1'
'aph
TTA AAT TCC AAC ... - 3'

FIG_12

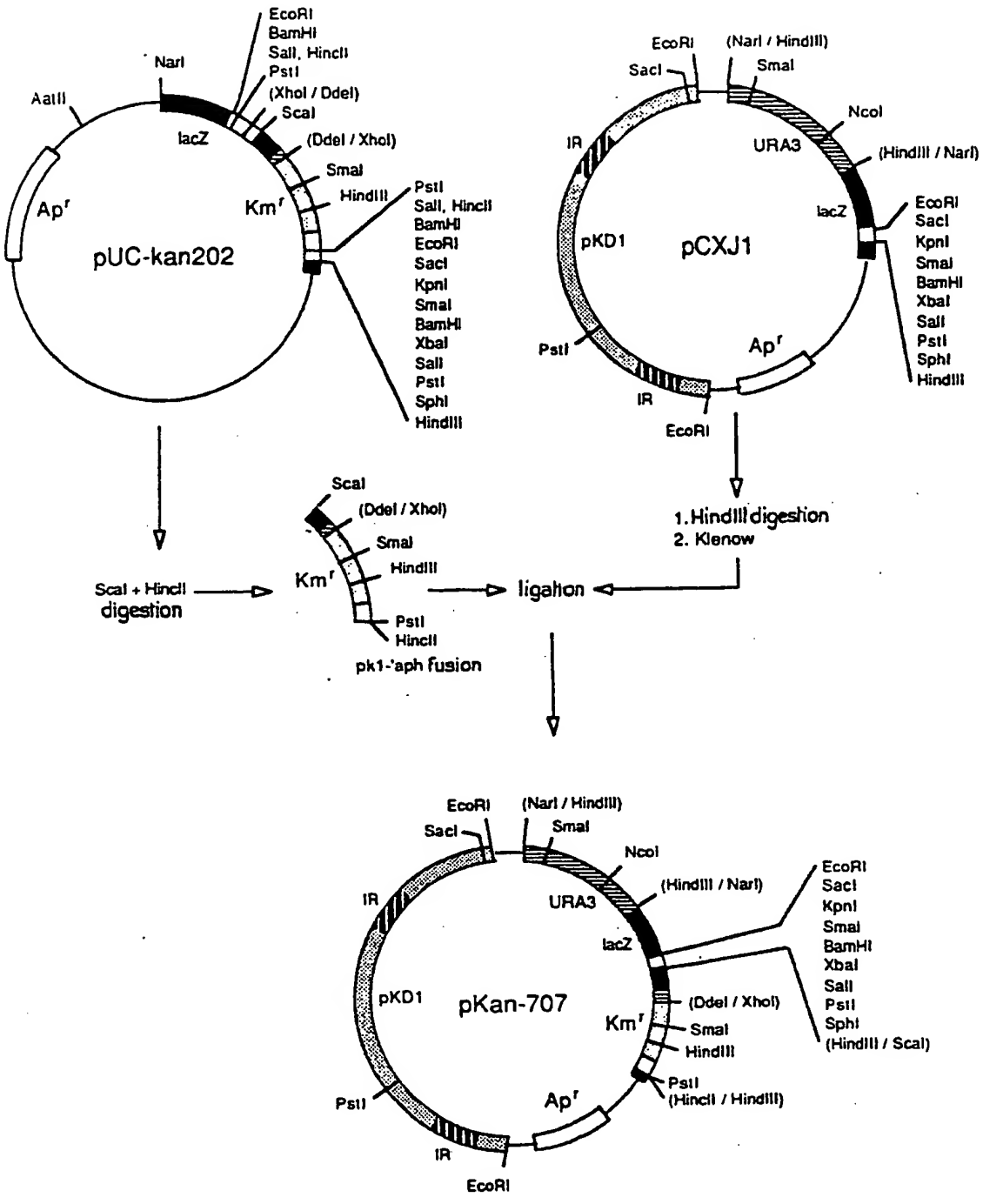

pUC-kan202
Ap^r
Km^r
lacZ
AatII
NarI
EcoRI
BamHI
SalI, HincII
PstI
(XhoI / DdeI)
ScaI
(DdeI / XhoI)
SmaI
HindIII
PstI
SalI, HincII
BamHI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
pCXJ1
pKD1
IR
URA3
lacZ
Ap^r
EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
(HindIII / NarI)
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
PstI
IR
EcoRI
1. HindIII digestion
2. Klenow
ligation
ScaI + HincII
digestion
ScaI
(DdeI / XhoI)
SmaI
HindIII
PstI
HincII
pk1-'aph fusion
pKan-707
pKD1
IR
URA3
lacZ
Km^r
Ap^r
EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
(HindIII / NarI)
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
(HindIII / ScaI)
(DdeI / XhoI)
SmaI
HindIII
PstI
(HincII / HindIII)
PstI
IR
EcoRI

FIG_13

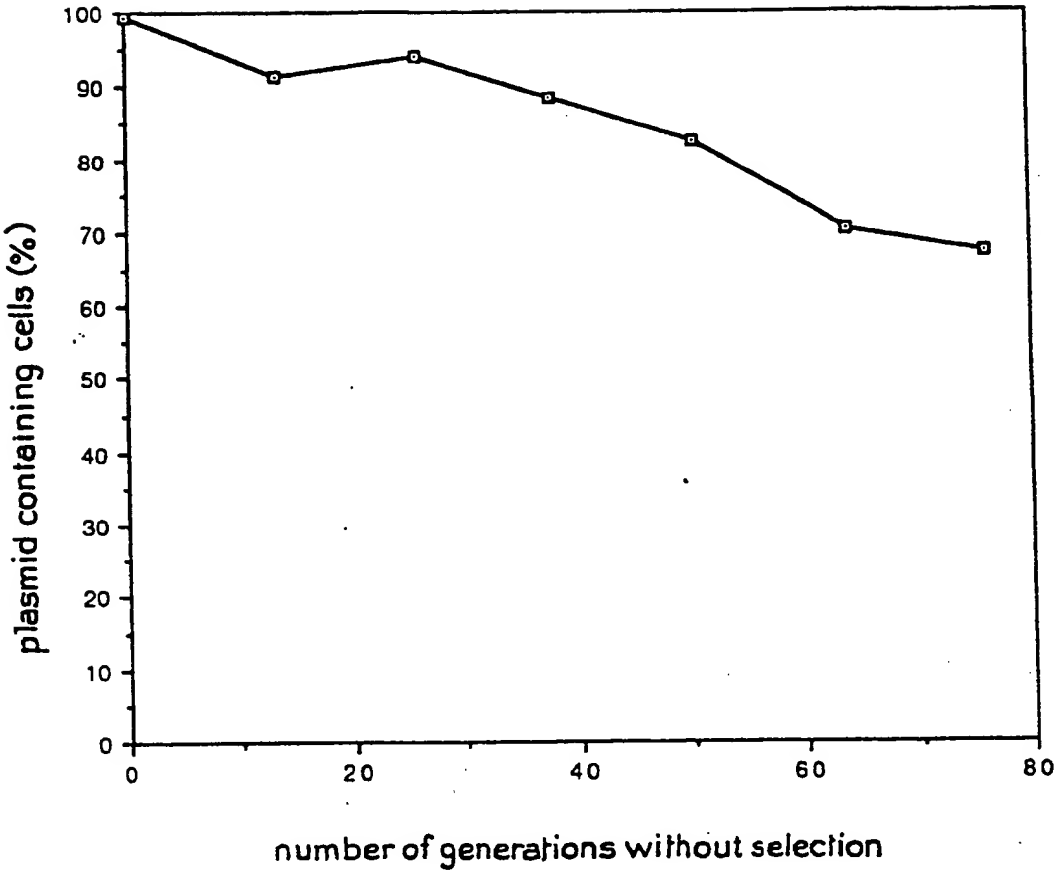

plasmid containing cells (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
number of generations without selection

FIG_14

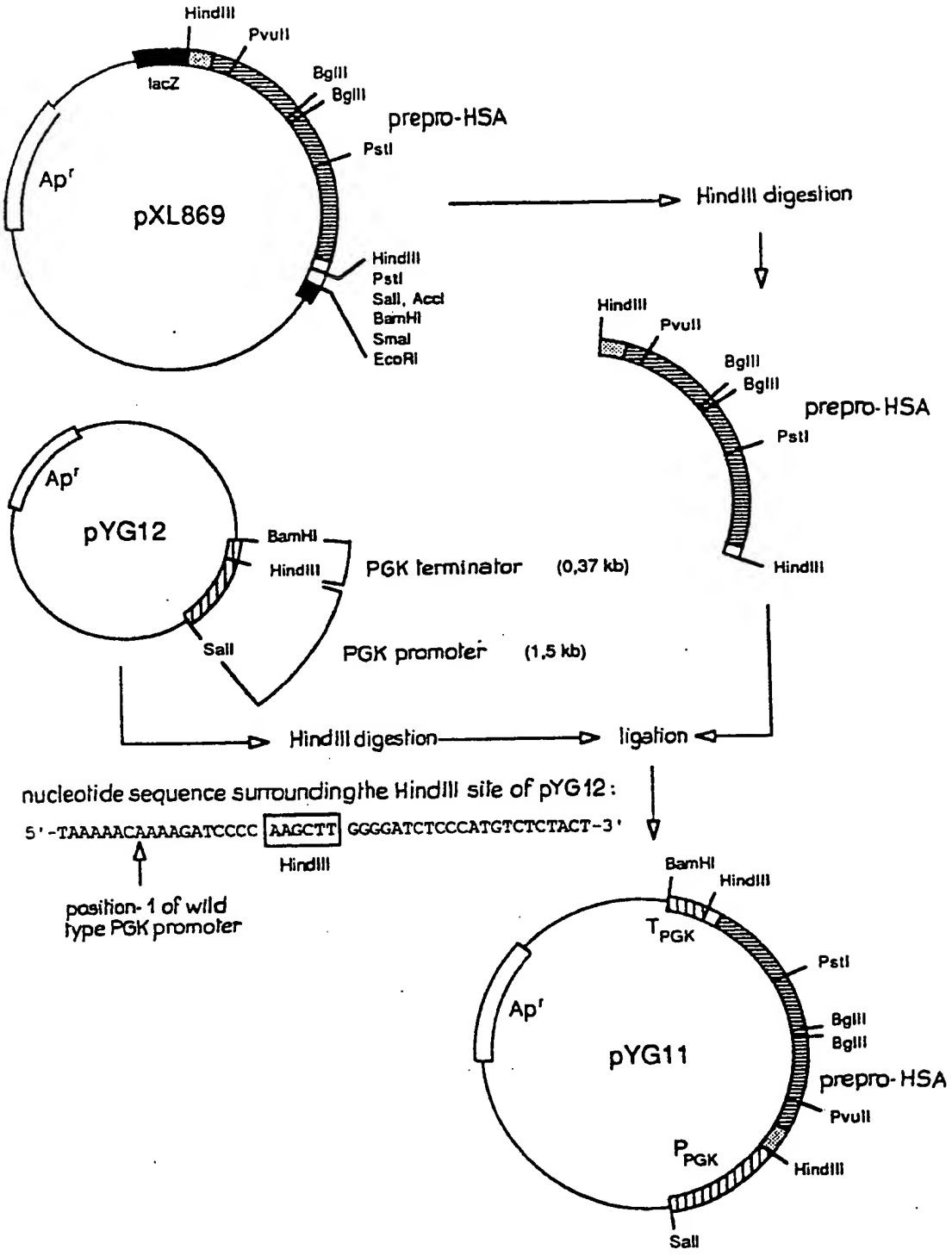

HindIII
PvuII
lacZ
BglII
BglII
prepro-HSA
PstI
Ap^r
pXL869
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
HindIII digestion
HindIII
PvuII
BglII
BglII
prepro-HSA
PstI
HindIII
Ap^r
pYG12
BamHI
HindIII
PGK terminator (0,37 kb)
SalI
PGK promoter (1,5 kb)
HindIII digestion
ligation
nucleotide sequence surrounding the HindIII site of pYG12:
5'-TAAAAACAAAAGATCCCC AAGCTT GGGGATCTCCCATGTCTCTACT-3'
HindIII
position-1 of wild type PGK promoter
BamHI
HindIII
T PGK
PstI
Ap^r
pYG11
BglII
BglII
prepro-HSA
PvuII
P PGK
HindIII
SalI

FIG.15

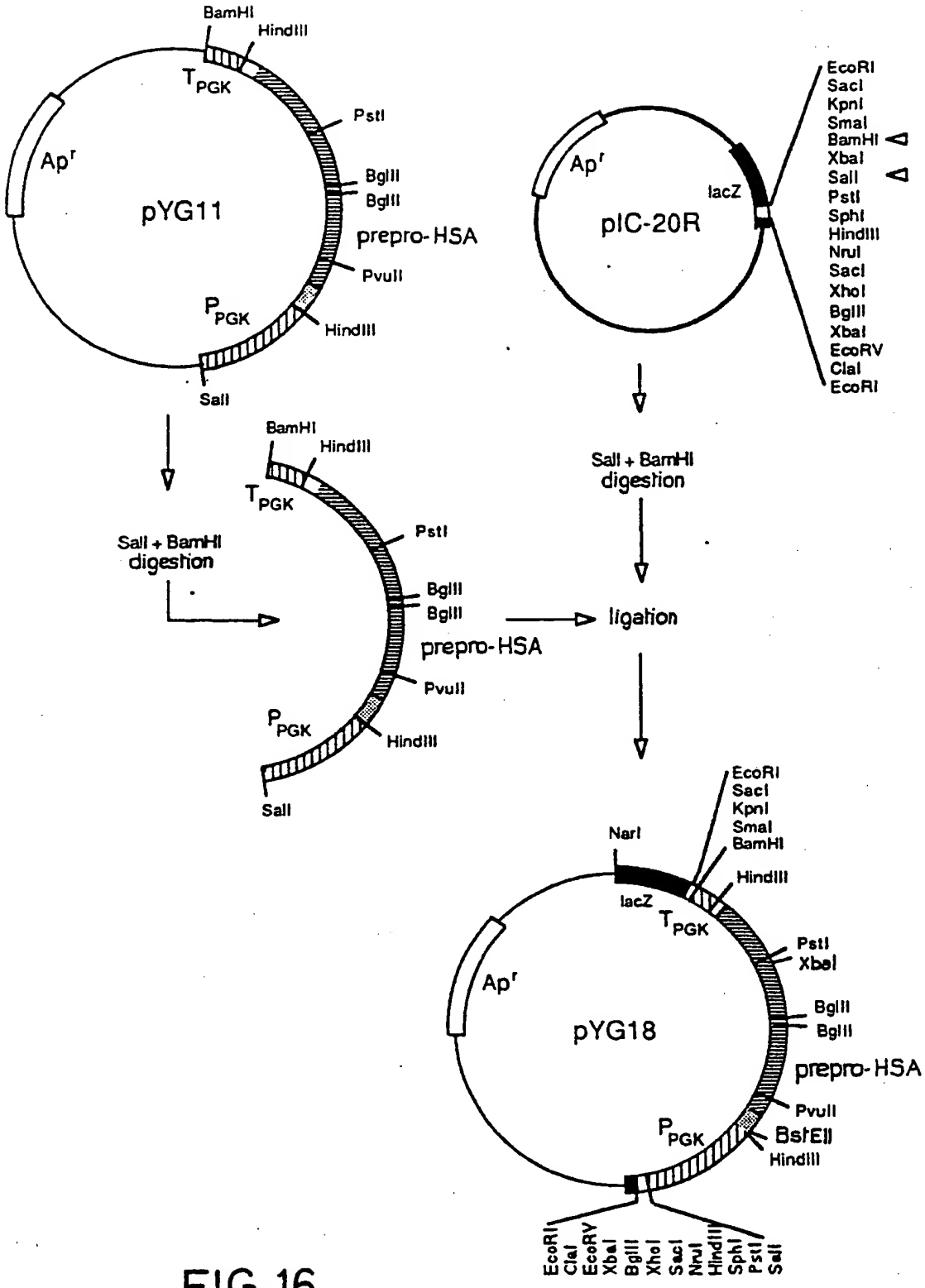

BamHI
HindIII
T PGK
PstI
Ap r
pYG11
BglII
BglII
prepro-HSA
PvuII
P PGK
HindIII
SalI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
NruI
SacI
XhoI
BglII
XbaI
EcoRV
ClaI
EcoRI
Ap r
lacZ
pIC-20R
SalI + BamHI
digestion
SalI + BamHI
digestion
ligation
NarI
lacZ
T PGK
Ap r
pYG18
P PGK
BstEII
EcoRI
ClaI
EcoRV
XbaI
BglII
XhoI
SacI
NruI
HindIII
SphI
PstI
SalI

FIG_16

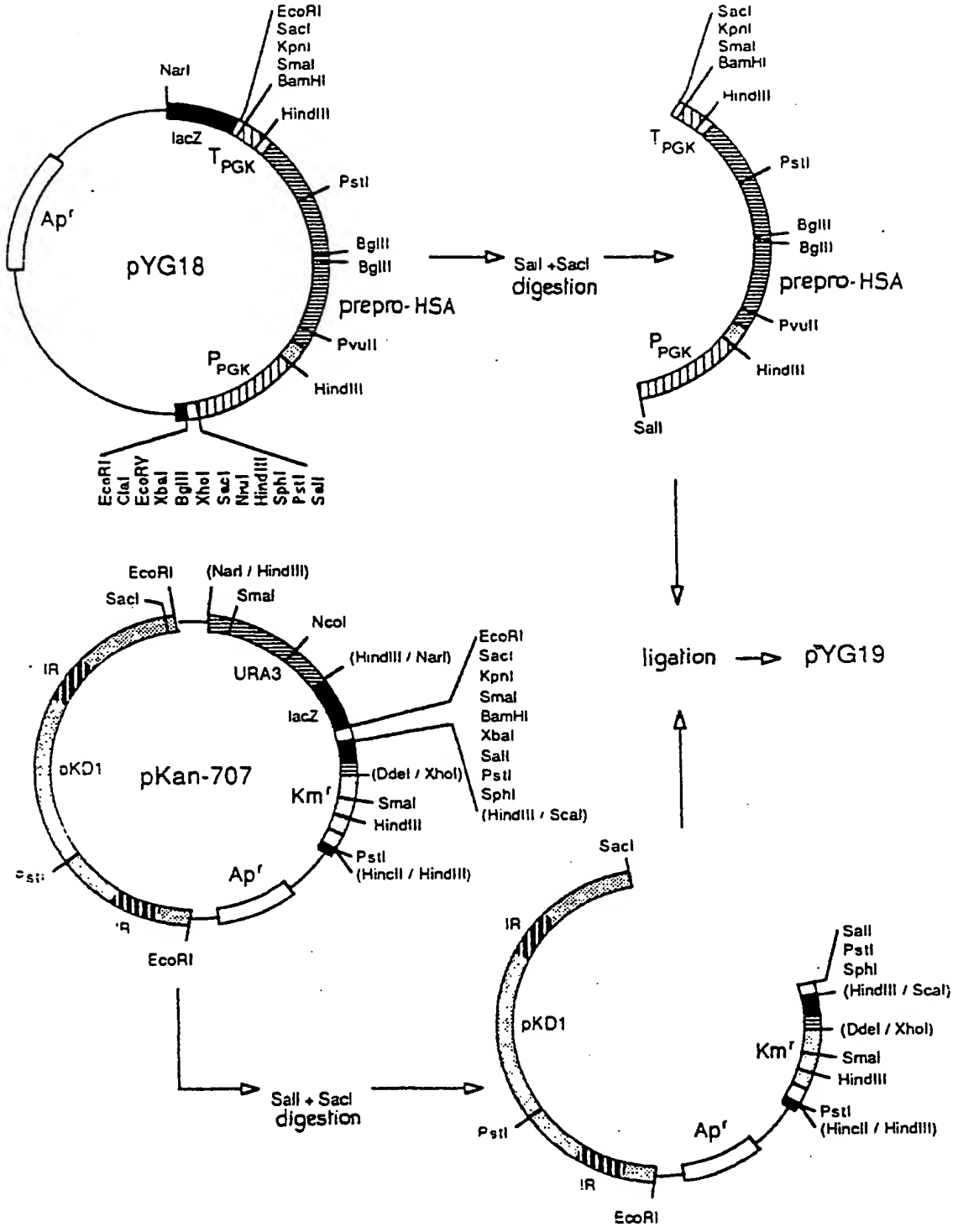
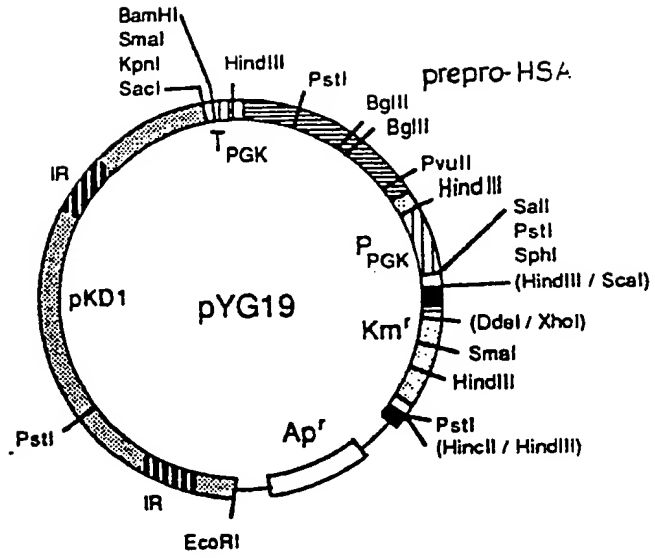
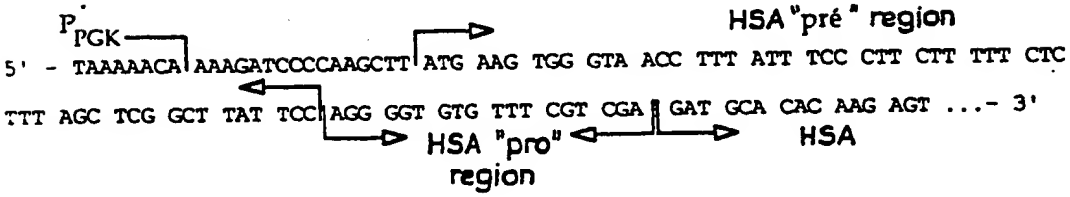
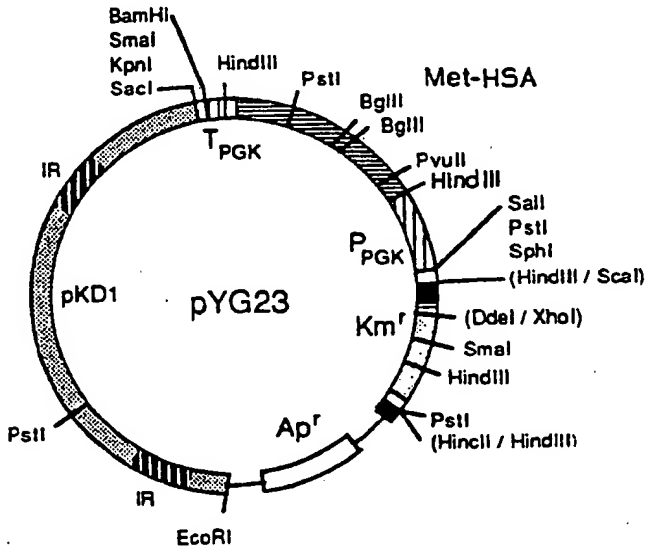

EcoRI
SacI
KpnI
SmaI
BamHI
NarI
HindIII
lacZ
T_{PGK}
PstI
Ap^r
pYG18
BglII
BglII
prepro-HSA
PvuII
P_{PGK}
HindIII
EcoRI ClaI EcoRV XbaI BglII XhoI SacI NruI HindIII SphI PstI SalI
SalI + SacI digestion
SacI
KpnI
SmaI
BamHI
HindIII
T_{PGK}
PstI
BglII
BglII
prepro-HSA
PvuII
P_{PGK}
HindIII
SalI
ligation
pYG19
EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
IR
URA3
(HindIII / NarI)
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
(HindIII / ScaI)
lacZ
pKD1
pKan-707
(DdeI / XhoI)
Km^r
SmaI
HindIII
PstI
(HincII / HindIII)
Ap^r
EcoRI
SacI
IR
SalI
PstI
SphI
(HindIII / ScaI)
pKD1
(DdeI / XhoI)
Km^r
SmaI
HindIII
PstI
(HincII / HindIII)
SalI + SacI digestion
PstI
Ap^r
IR
EcoRI

FIG_17


BamHI
SmaI
KpnI
SacI
HindIII
PstI
prepro-HSA
BglII
BglII
T_{PGK}
IR
PvuII
HindIII
SalI
PstI
SphI
(HindIII / ScaI)
P_{PGK}
pKD1
pYG19
Km^r
(DdeI / XhoI)
SmaI
HindIII
PstI
(HincII / HindIII)
Ap^r
PstI
IR
EcoRI


P_{PGK}
HSA "pré" region
5' - TAAAAACA AAAGATCCCCAAGCTT ATG AAG TGG GTA ACC TTT ATT TCC CTT CTT TTT CTC
TTT AGC TCG GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA CAC AAG AGT ...- 3'
HSA "pro" region
HSA


BamHI
SmaI
KpnI
SacI
HindIII
PstI
Met-HSA
BglII
BglII
T_{PGK}
IR
PvuII
HindIII
SalI
PstI
SphI
(HindIII / ScaI)
P_{PGK}
pKD1
pYG23
Km^r
(DdeI / XhoI)
SmaI
HindIII
PstI
(HincII / HindIII)
Ap^r
PstI
IR
EcoRI


P_{PGK}
5' - TAAAAACA AAAGATCCCCAAGCTT ATG GAT GCA CAC AAG AGT ...- 3'
Met-HSA

FIG_18

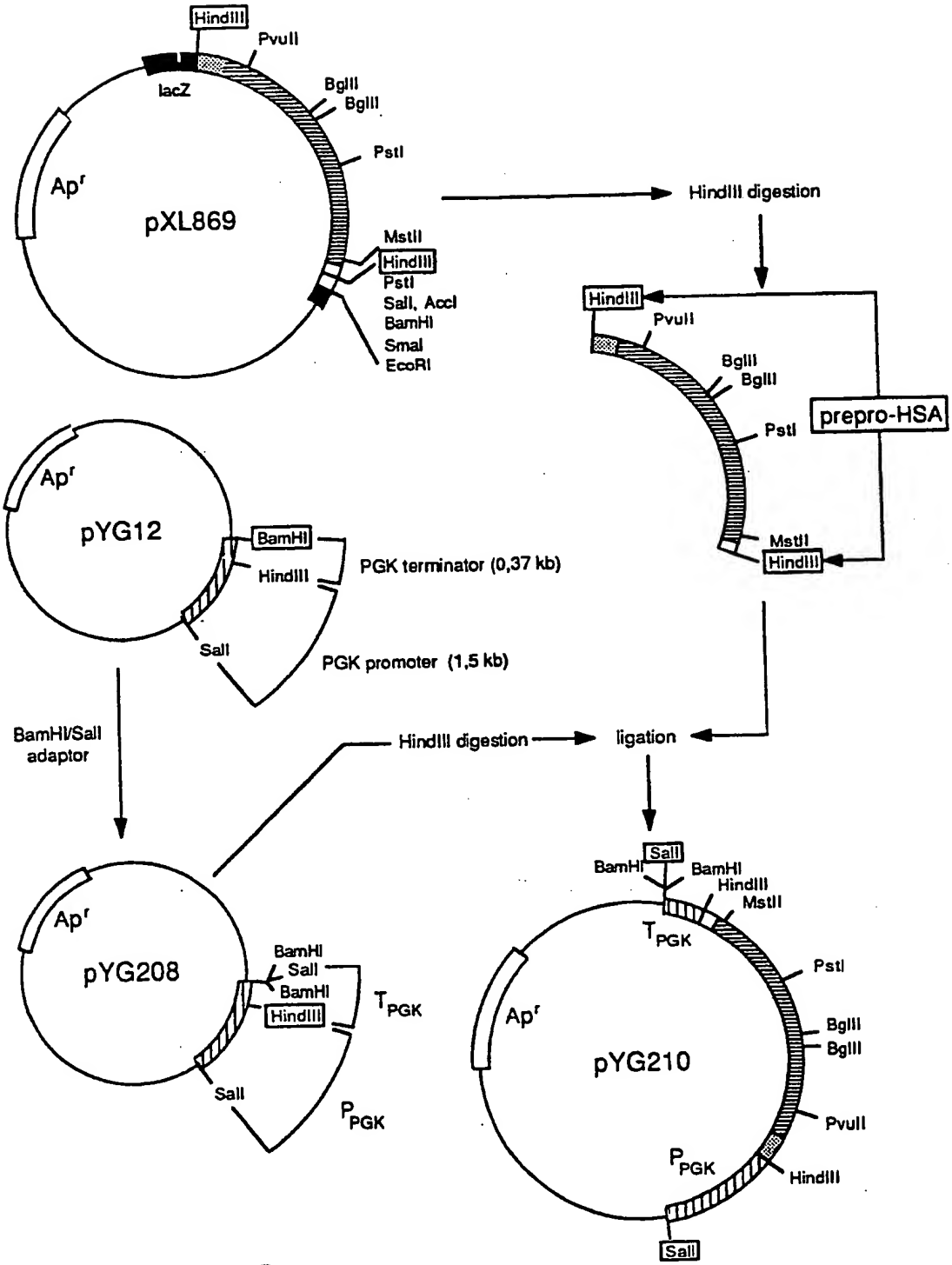

HindIII
PvuII
lacZ
BglII
BglII
PstI
Ap^r
pXL869
MstII
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
HindIII digestion
HindIII
PvuII
BglII
BglII
PstI
prepro-HSA
MstII
HindIII
Ap^r
pYG12
BamHI
HindIII
PGK terminator (0,37 kb)
SalI
PGK promoter (1,5 kb)
BamHI/SalI adaptor
HindIII digestion
ligation
Ap^r
pYG208
BamHI
SalI
BamHI
HindIII
T_PGK
SalI
P_PGK
SalI
BamHI
BamHI
HindIII
MstII
T_PGK
PstI
Ap^r
pYG210
BglII
BglII
PvuII
P_PGK
HindIII
SalI

FIG_19A

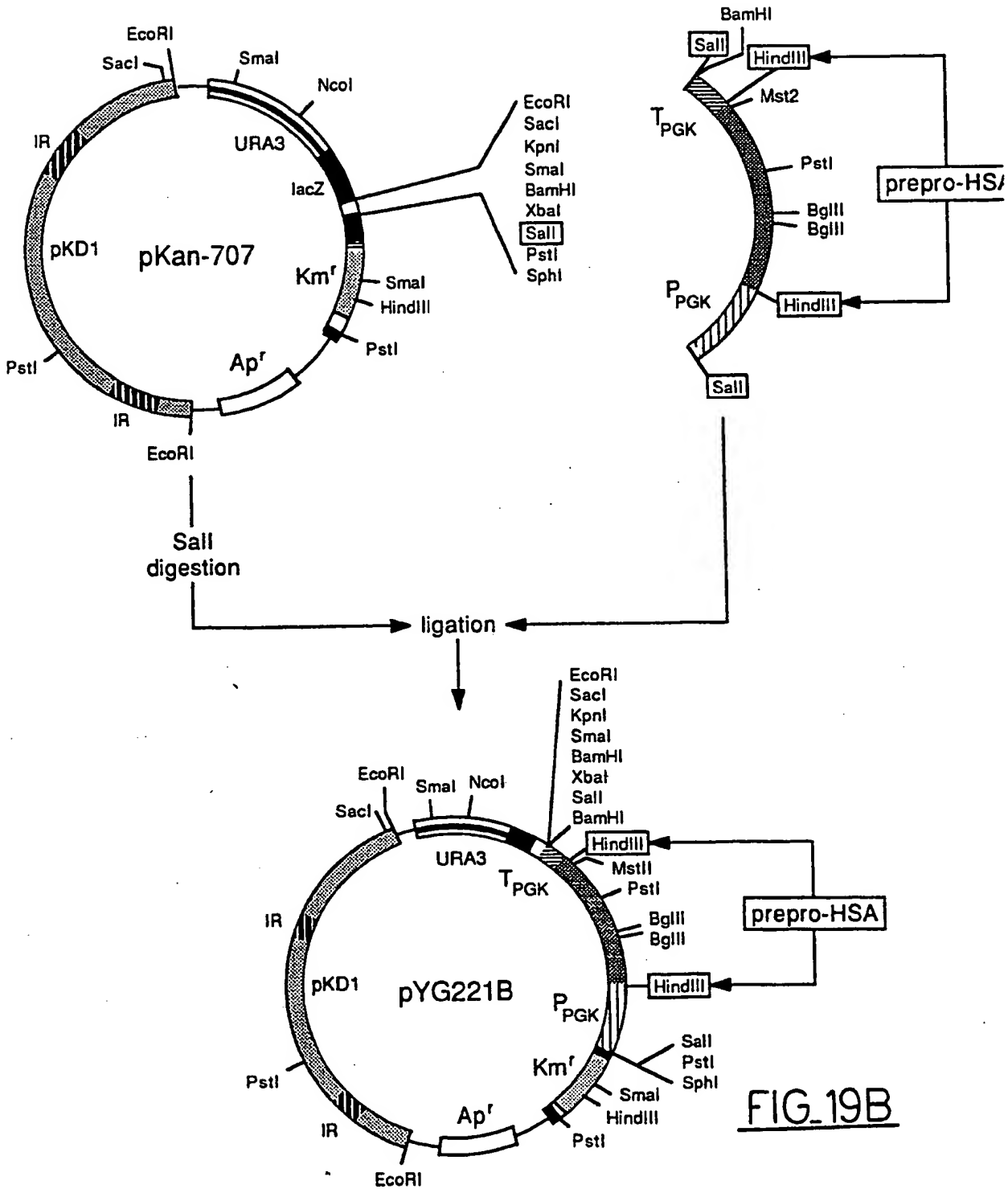

EcoRI
SacI
SmaI
NcoI
URA3
lacZ
IR
pKD1
pKan-707
Km^r
Ap^r
PstI
IR
EcoRI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
SmaI
HindIII
PstI
SalI
digestion
BamHI
SalI
HindIII
Mst2
T PGK
PstI
BglII
BglII
P PGK
HindIII
SalI
prepro-HSA
ligation
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
BamHI
EcoRI
SmaI
NcoI
SacI
URA3
HindIII
MstII
T PGK
PstI
IR
BglII
BglII
prepro-HSA
pKD1
pYG221B
HindIII
P PGK
SalI
PstI
SphI
Km^r
SmaI
HindIII
PstI
PstI
IR
Ap^r
EcoRI

FIG_19B

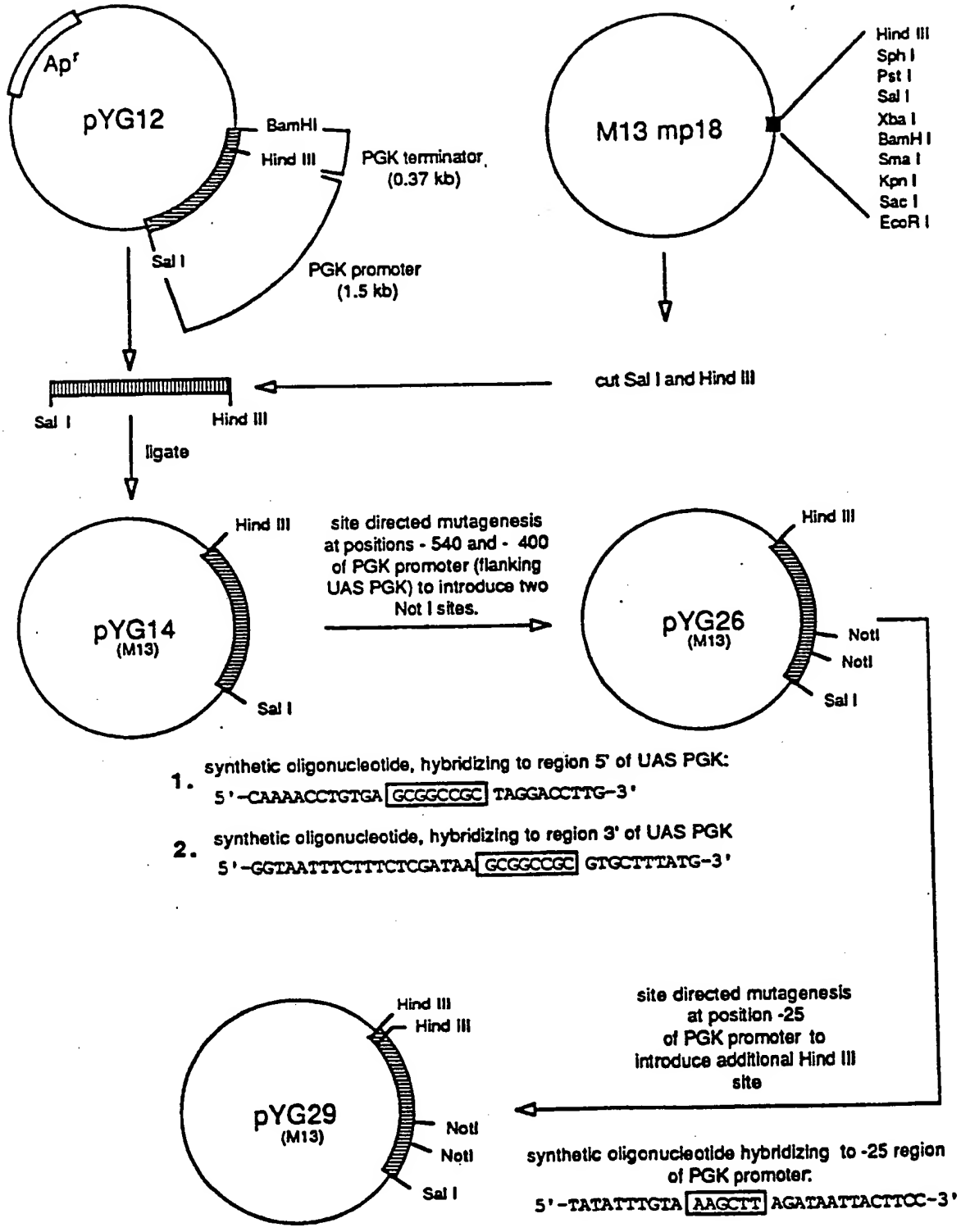
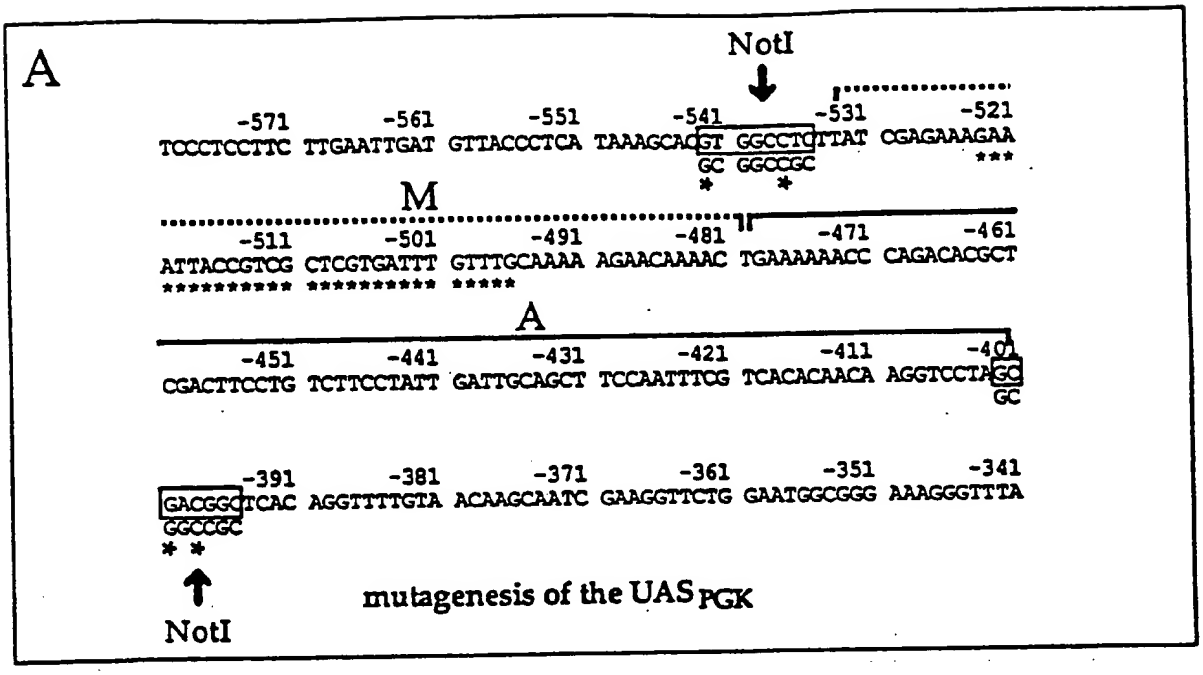

Ap^r
pYG12
BamHI
Hind III
PGK terminator
(0.37 kb)
Sal I
PGK promoter
(1.5 kb)
M13 mp18
Hind III
Sph I
Pst I
Sal I
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
cut Sal I and Hind III
Sal I
Hind III
ligate
pYG14
(M13)
Hind III
Sal I
site directed mutagenesis
at positions - 540 and - 400
of PGK promoter (flanking
UAS PGK) to introduce two
Not I sites.
pYG26
(M13)
Hind III
NotI
NotI
Sal I
1. synthetic oligonucleotide, hybridizing to region 5' of UAS PGK:
5'-CAAAACCTGTGA GCGGCCGC TAGGACCTTG-3'
2. synthetic oligonucleotide, hybridizing to region 3' of UAS PGK
5'-GGTAATTTCTTTCTCGATAA GCGGCCGC GTGCTTTATG-3'
site directed mutagenesis
at position -25
of PGK promoter to
introduce additional Hind III
site
pYG29
(M13)
Hind III
Hind III
NotI
NotI
Sal I
synthetic oligonucleotide hybridizing to -25 region
of PGK promoter.
5'-TATATTTGTA AAGCTT AGATAATTACTTCC-3'

FIG_20


A
NotI
-571 -561 -551 -541 -531 -521
TCCCTCCTTC TTGAATTGAT GTTACCCTCA TAAAGCACGT GGCCTCTTTAT CGAGAAAGAA
GC GGCCGC
M
-511 -501 -491 -481 -471 -461
ATTACCGTCG CTCGTGATTT GTTTGCAAAA AGAACAAAAC TGAAAAAACC CAGACACGCT
A
-451 -441 -431 -421 -411 -401
CGACTTCCTG TCTTCCTATT GATTGCAGCT TCCAATTTCG TCACACAACA AGGTCCTAGC
GC
-391 -381 -371 -361 -351 -341
GACGGCTCAC AGGTTTTGTA ACAAGCAATC GAAGGTTCTG GAATGGCGGG AAAGGGTTTA
GGCCGC
NotI
mutagenesis of the UAS PGK

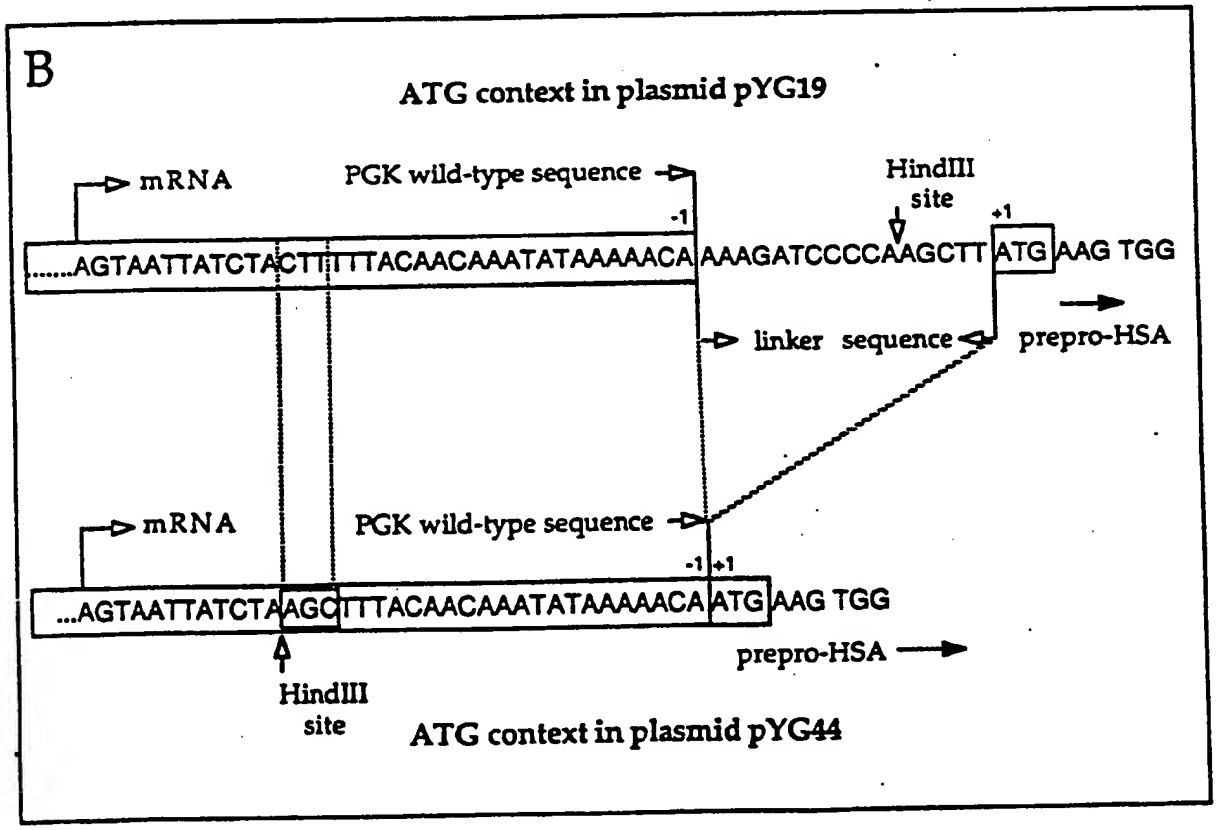

B
ATG context in plasmid pYG19
mRNA
PGK wild-type sequence
-1
HindIII site
+1
.....AGTAATTATCTACTTTTTACAACAAATATAAAAACA AAAGATCCCCAAGCTT ATG AAG TGG
linker sequence
prepro-HSA
mRNA
PGK wild-type sequence
-1 +1
...AGTAATTATCTAAGCTTTACAACAAATATAAAAACA ATG AAG TGG
prepro-HSA
HindIII site
ATG context in plasmid pYG44

FIG_21

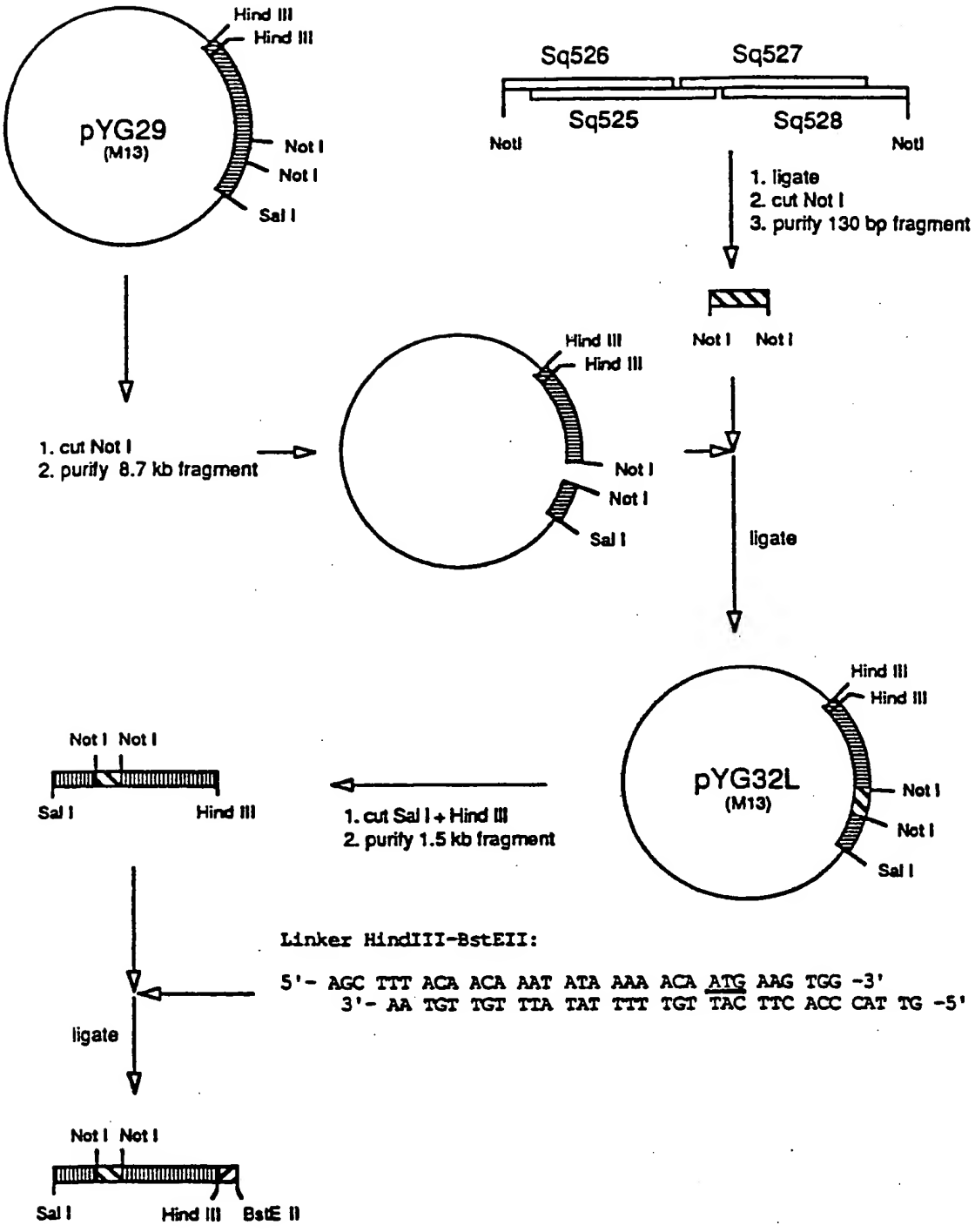

Hind III
Hind III
pYG29
(M13)
Not I
Not I
Sal I
Sq526
Sq527
Sq525
Sq528
NotI
NotI
1. ligate
2. cut Not I
3. purify 130 bp fragment
Not I
Not I
1. cut Not I
2. purify 8.7 kb fragment
Hind III
Hind III
Not I
Not I
Sal I
ligate
Hind III
Hind III
pYG32L
(M13)
Not I
Not I
Sal I
Not I Not I
Sal I
Hind III
1. cut Sal I + Hind III
2. purify 1.5 kb fragment
Linker HindIII-BstEII:
5'- AGC TTT ACA ACA AAT ATA AAA ACA ATG AAG TGG -3'
3'- AA TGT TGT TTA TAT TTT TGT TAC TTC ACC CAT TG -5'
ligate
Not I Not I
Sal I
Hind III
BstE II

FIG_22

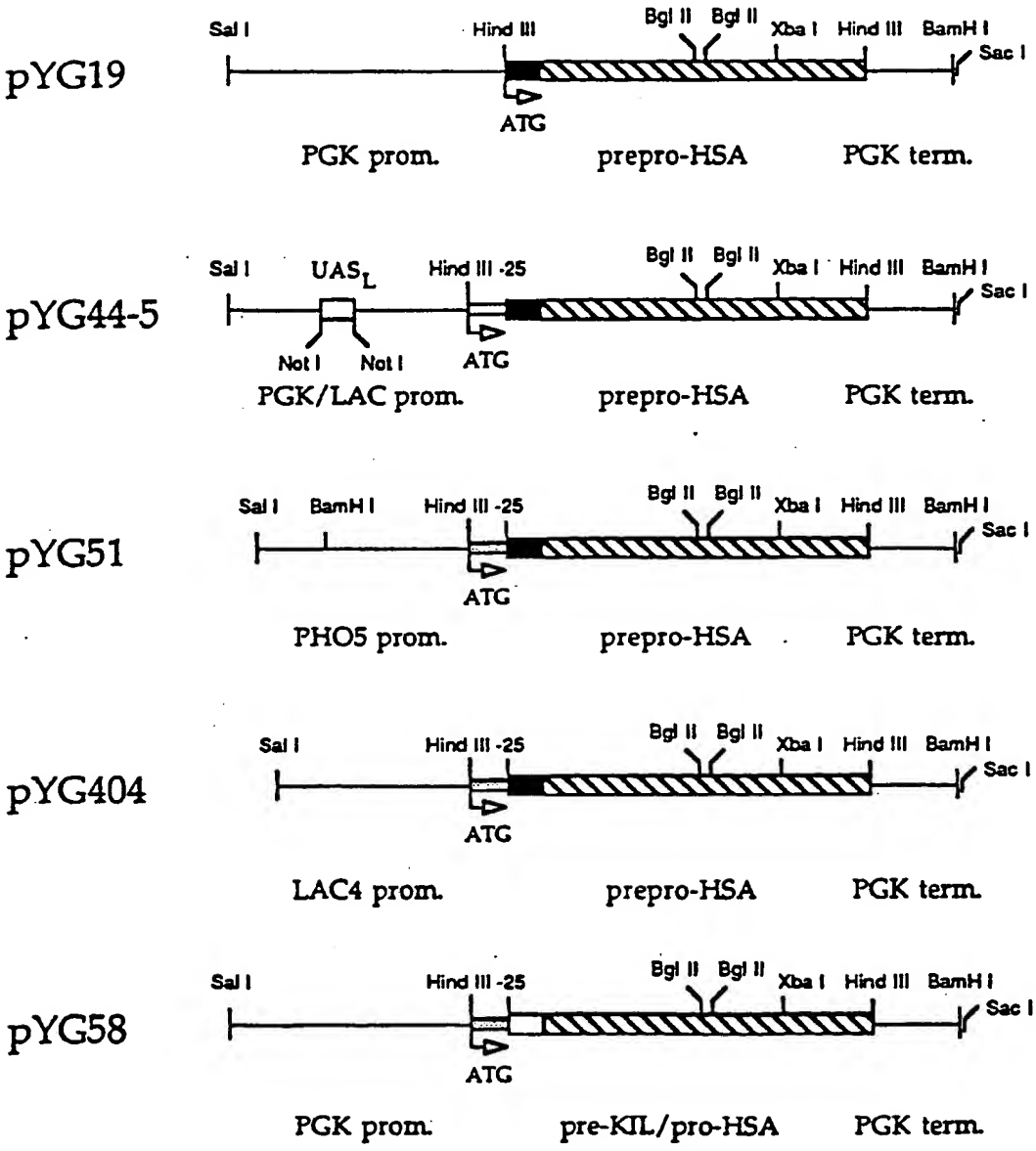

pYG19
Sal I
Hind III
Bgl II
Bgl II
Xba I
Hind III
BamH I
Sac I
ATG
PGK prom.
prepro-HSA
PGK term.
pYG44-5
Sal I
UAS$_L$
Hind III -25
Bgl II
Bgl II
Xba I
Hind III
BamH I
Sac I
Not I
Not I
ATG
PGK/LAC prom.
prepro-HSA
PGK term.
pYG51
Sal I
BamH I
Hind III -25
Bgl II
Bgl II
Xba I
Hind III
BamH I
Sac I
ATG
PHO5 prom.
prepro-HSA
PGK term.
pYG404
Sal I
Hind III -25
Bgl II
Bgl II
Xba I
Hind III
BamH I
Sac I
ATG
LAC4 prom.
prepro-HSA
PGK term.
pYG58
Sal I
Hind III -25
Bgl II
Bgl II
Xba I
Hind III
BamH I
Sac I
ATG
PGK prom.
pre-KIL/pro-HSA
PGK term.

FIG_23

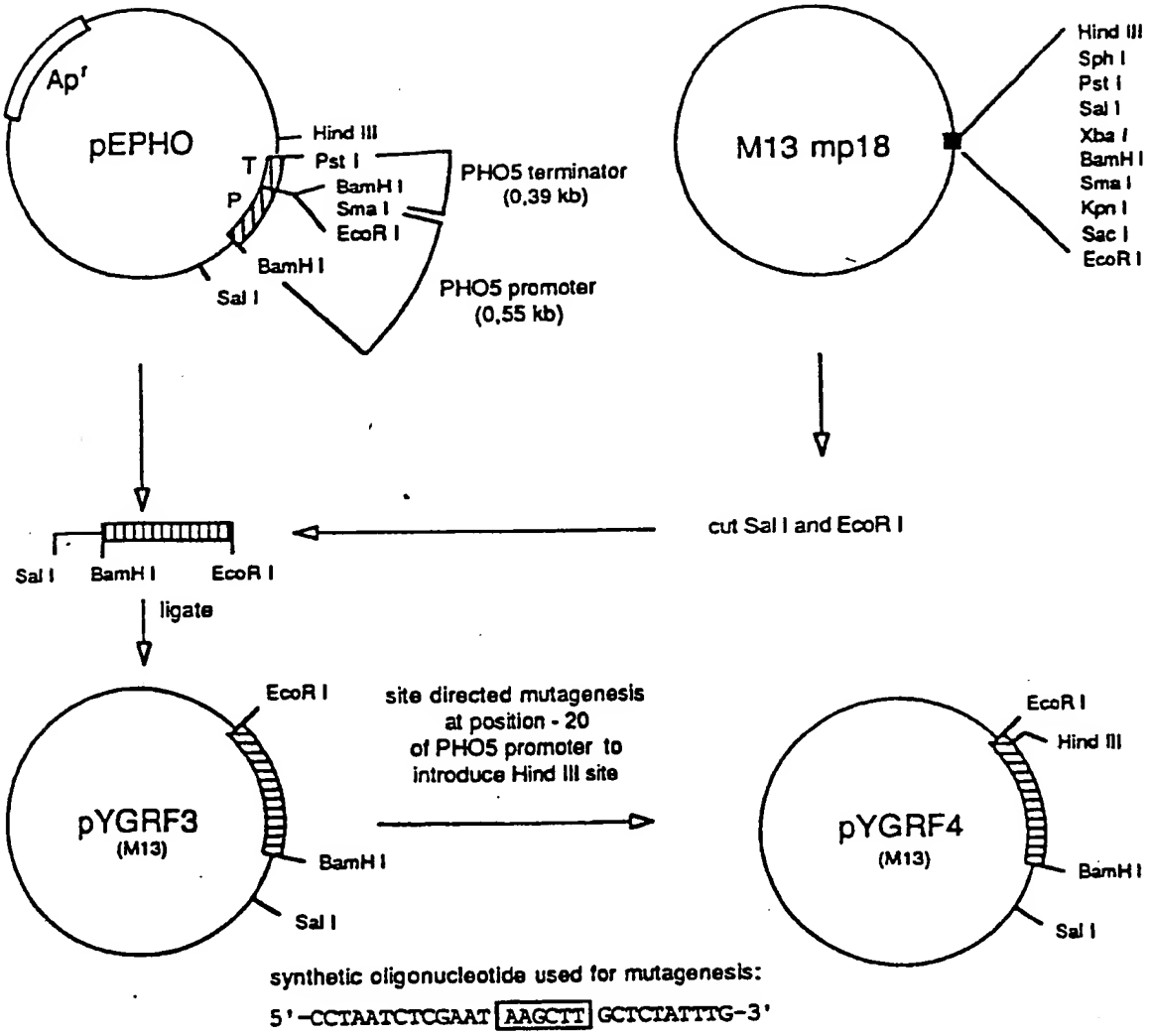

Ap^r
pEPHO
T
P
Hind III
Pst I
BamH I
Sma I
EcoR I
BamH I
Sal I
PHO5 terminator
(0,39 kb)
PHO5 promoter
(0,55 kb)
M13 mp18
Hind III
Sph I
Pst I
Sal I
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
cut Sal I and EcoR I
Sal I
BamH I
EcoR I
ligate
EcoR I
pYGRF3
(M13)
BamH I
Sal I
site directed mutagenesis
at position - 20
of PHO5 promoter to
introduce Hind III site
EcoR I
Hind III
pYGRF4
(M13)
BamH I
Sal I
synthetic oligonucleotide used for mutagenesis:
5'-CCTAATCTCGAAT AAGCTT GCTCTATTTG-3'

FIG. 24

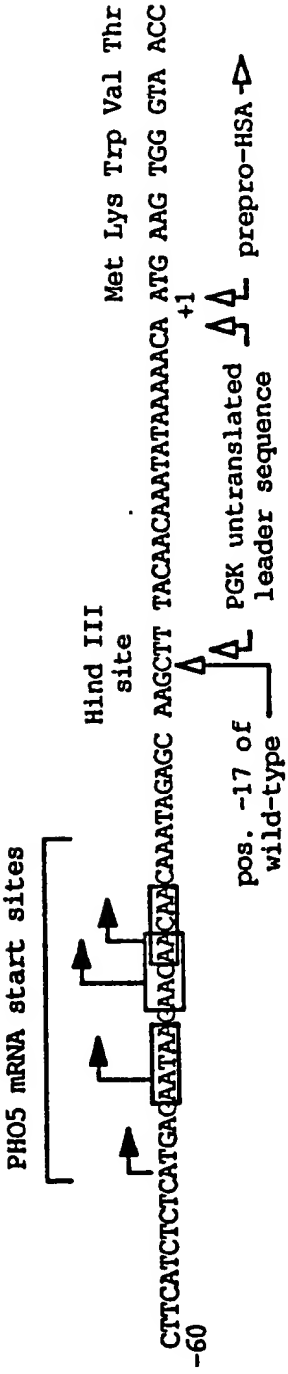

PHO5 mRNA start sites
Hind III site
Met Lys Trp Val Thr
CTTCATCTCTCATGACAATAACAACACAAATAGAGC AAGCTT TACAACAAATATAAAACA ATG AAG TGG GTA ACC
-60
+1
pos. -17 of wild-type PHO5 promoter
PGK untranslated leader sequence
prepro-HSA

FIG. 25

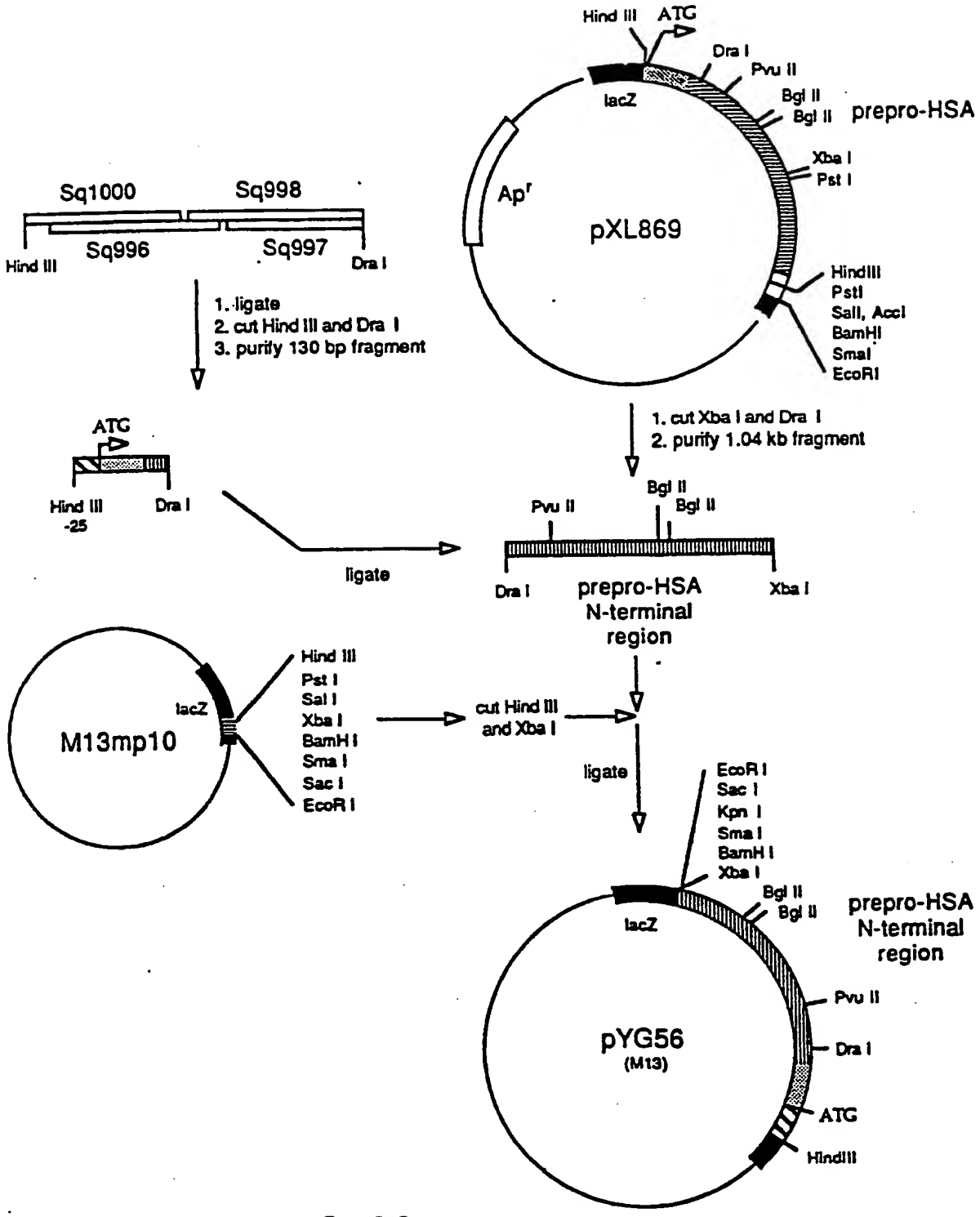

Sq1000
Sq998
Sq996
Sq997
Hind III
Dra I
1. ligate
2. cut Hind III and Dra I
3. purify 130 bp fragment
ATG
Hind III
-25
Dra I
ligate
Hind III
ATG
Dra I
Pvu II
Bgl II
lacZ
Bgl II
prepro-HSA
Xba I
Pst I
Ap^r
pXL869
HindIII
PstI
SalI, AccI
BamHI
SmaI
EcoRI
1. cut Xba I and Dra I
2. purify 1.04 kb fragment
Bgl II
Pvu II
Bgl II
Dra I
prepro-HSA
N-terminal
region
Xba I
M13mp10
lacZ
Hind III
Pst I
Sal I
Xba I
BamH I
Sma I
Sac I
EcoR I
cut Hind III
and Xba I
ligate
EcoR I
Sac I
Kpn I
Sma I
BamH I
Xba I
Bgl II
Bgl II
lacZ
prepro-HSA
N-terminal
region
Pvu II
pYG56
(M13)
Dra I
ATG
HindIII

FIG_26

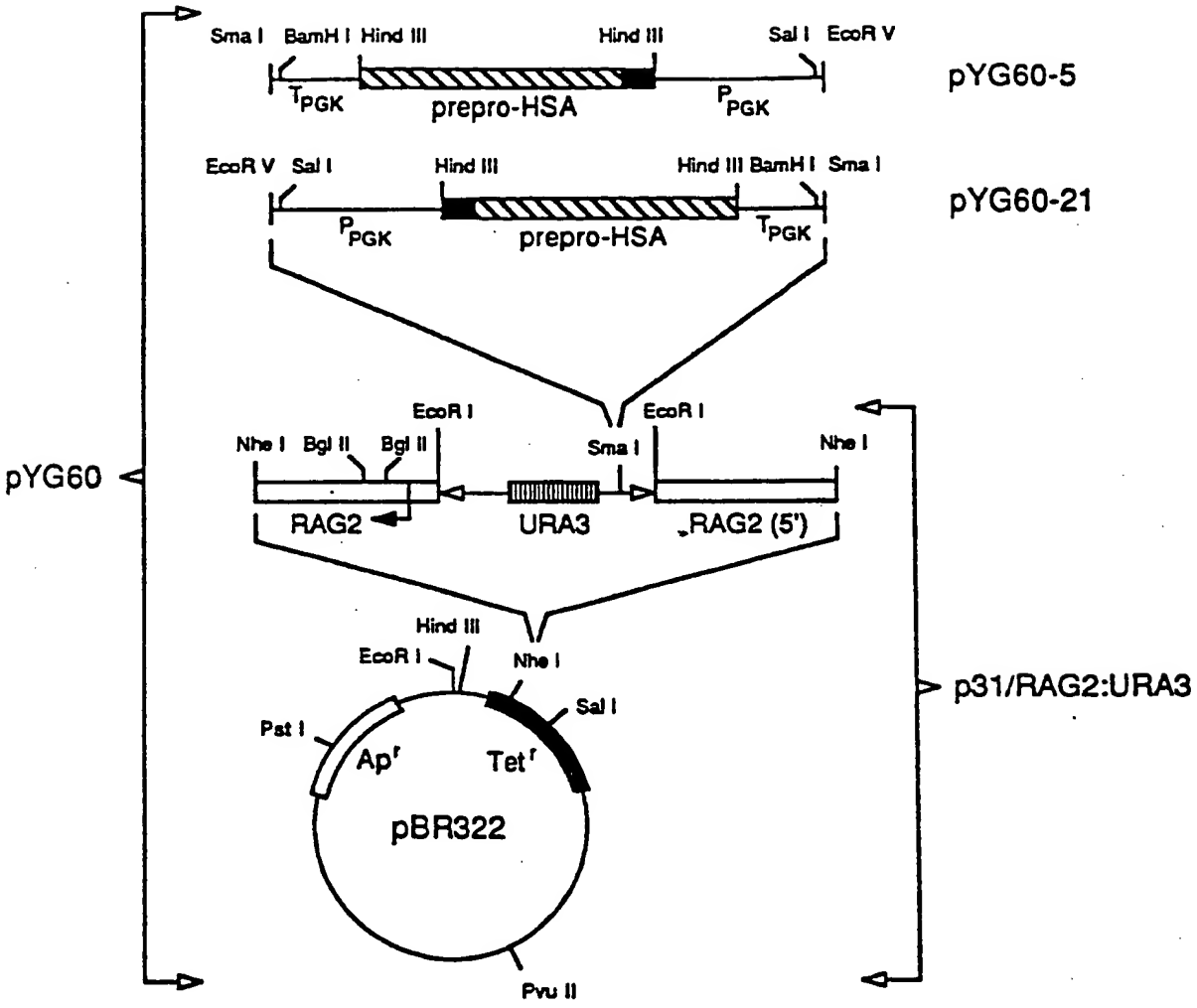

Sma I
BamH I
Hind III
Hind III
Sal I
EcoR V
pYG60-5
T PGK
prepro-HSA
P PGK
EcoR V
Sal I
Hind III
Hind III
BamH I
Sma I
pYG60-21
P PGK
prepro-HSA
T PGK
EcoR I
EcoR I
Nhe I
Bgl II
Bgl II
Sma I
Nhe I
pYG60
RAG2
URA3
RAG2 (5')
Hind III
EcoR I
Nhe I
Sal I
Pst I
Ap r
Tet r
pBR322
Pvu II
p31/RAG2:URA3

FIG. 27

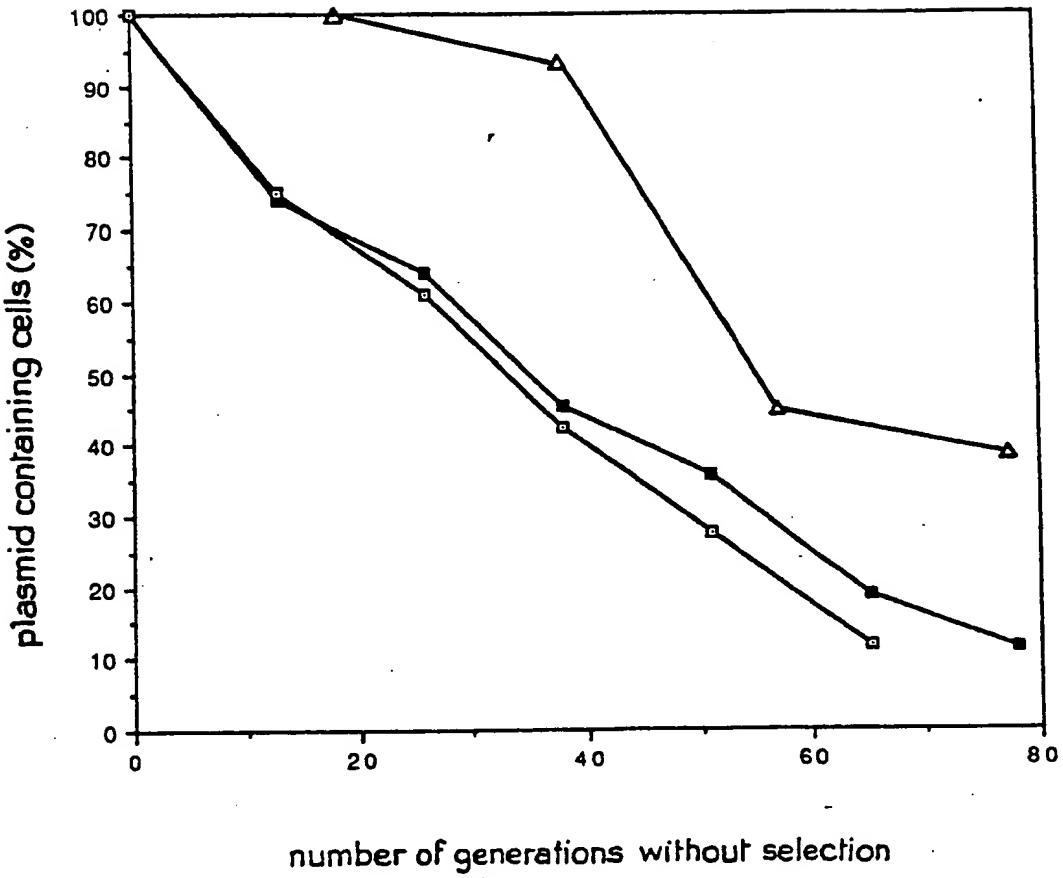

plasmid containing cells (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
number of generations without selection

FIG_28

FIG_29

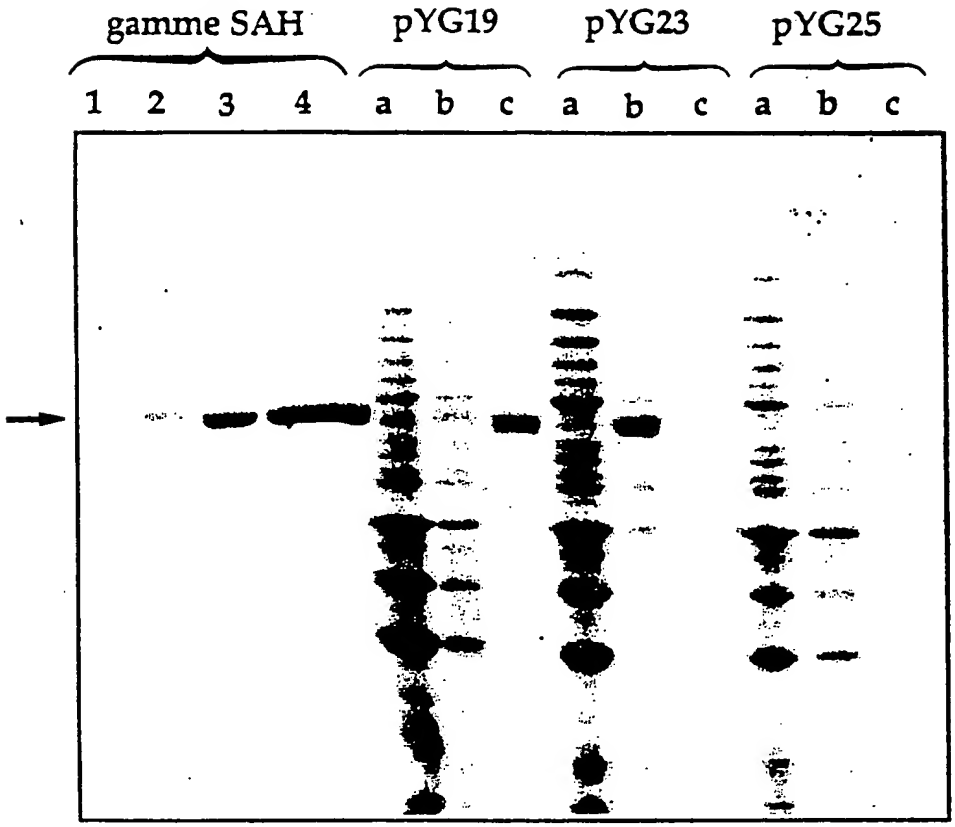
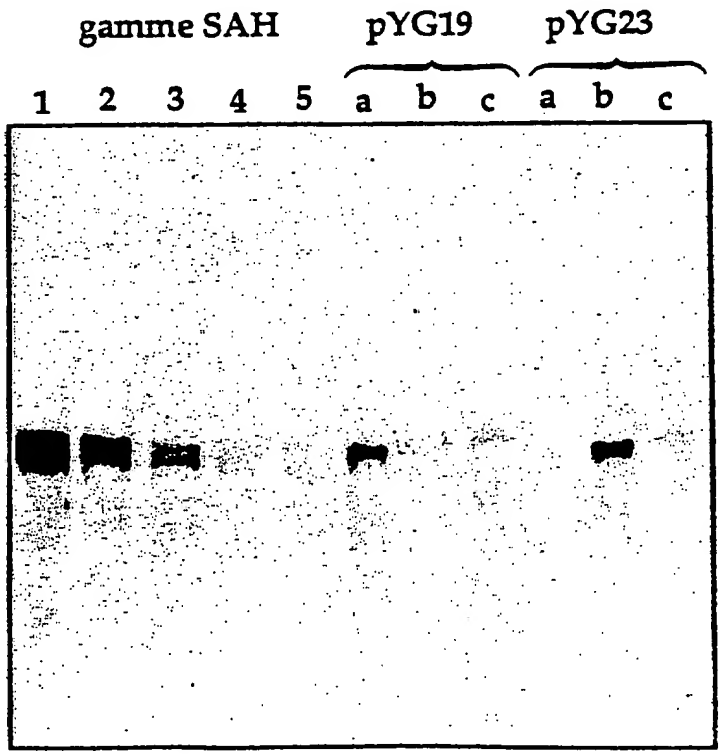

gamme SAH
pYG19
pYG23
pYG25
1 2 3 4
a b c a b c a b c

FIG_30


gamme SAH
pYG19
pYG23
1 2 3 4 5
a b c a b c

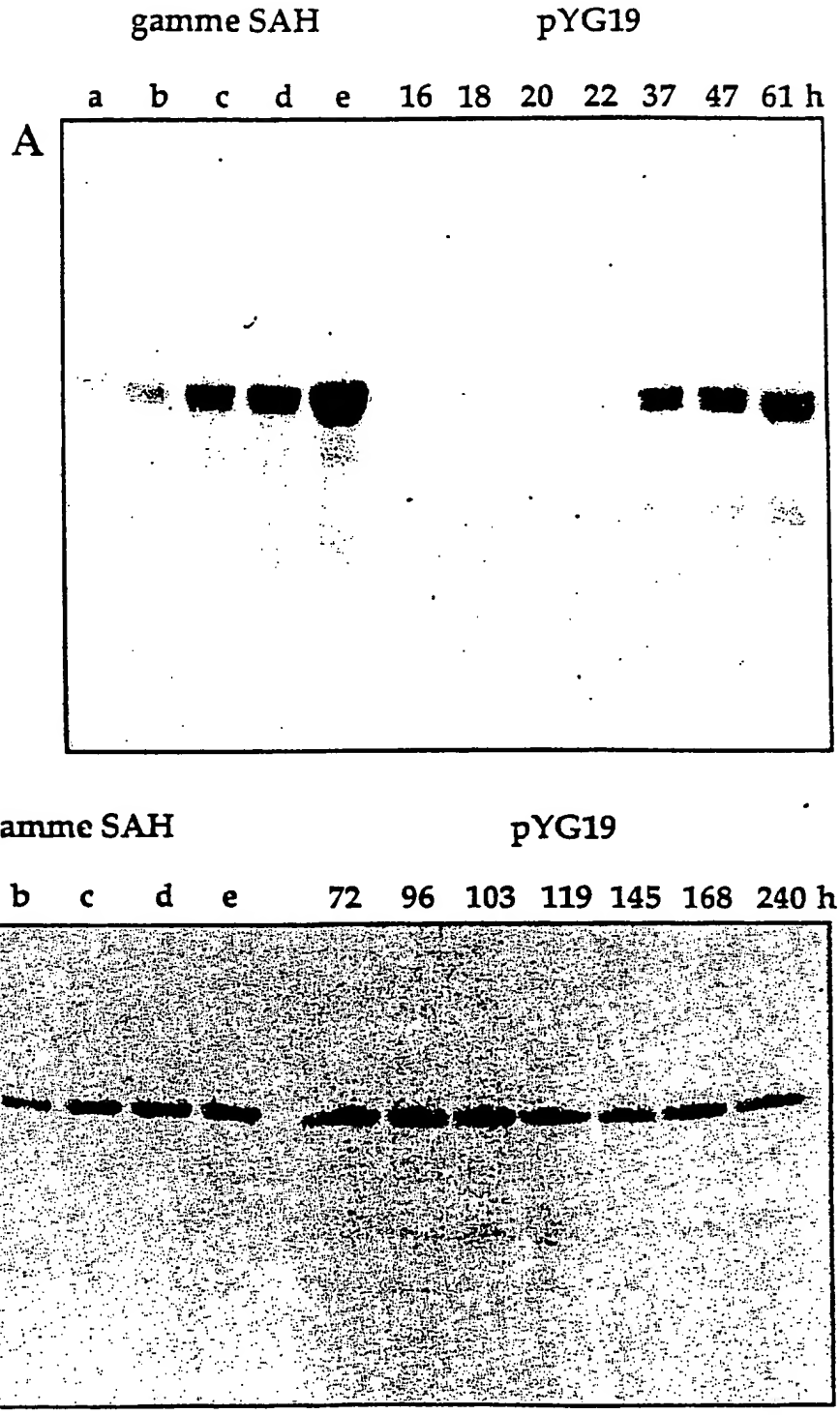

gamme SAH
pYG19
a b c d e 16 18 20 22 37 47 61 h
A
gamme SAH
pYG19
a b c d e 72 96 103 119 145 168 240 h
B

FIG_31

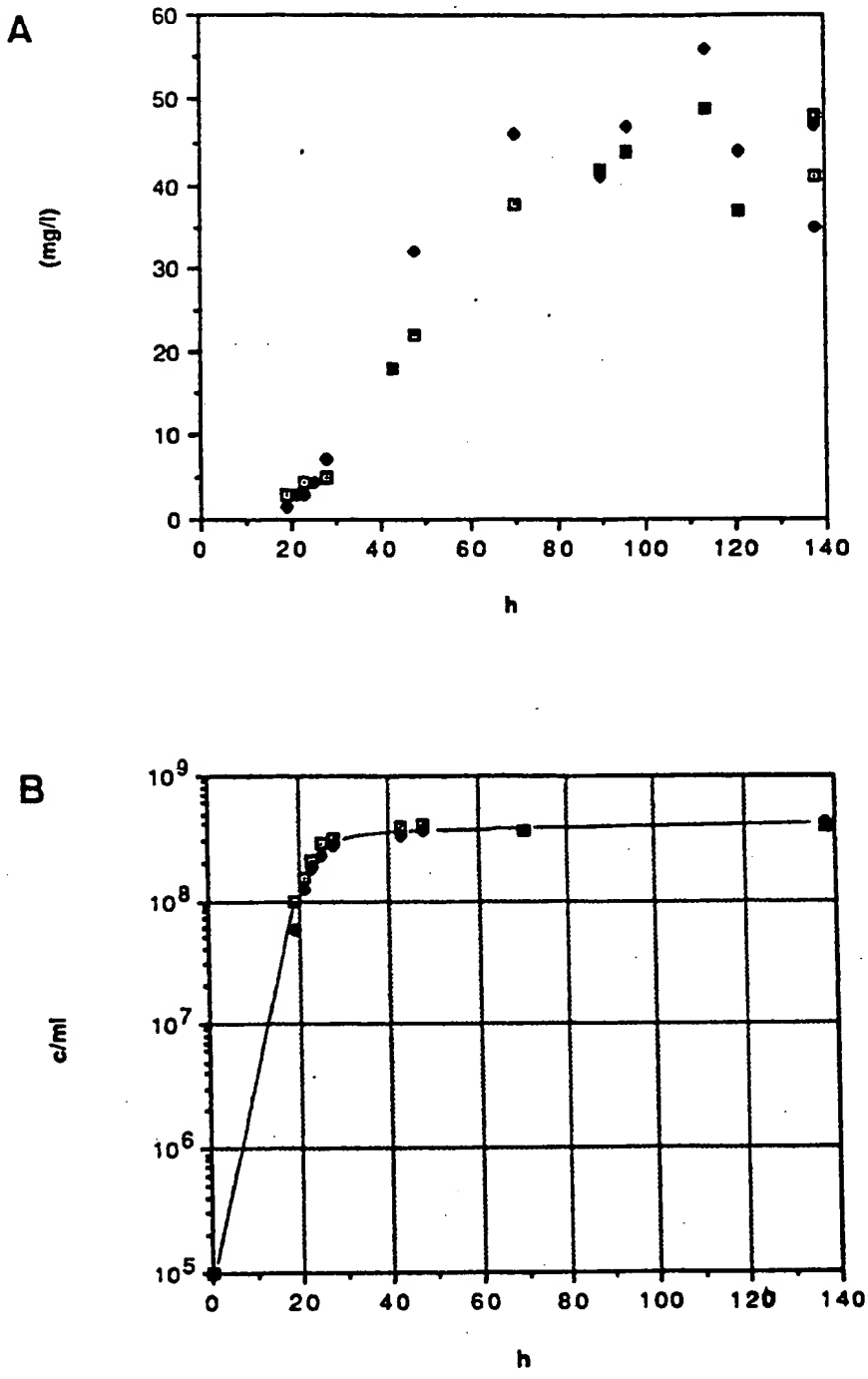

A
(mg/l)
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
h
B
c/ml
10^9
10^8
10^7
10^6
10^5
0
20
40
60
80
100
120
140
h

FIG_32

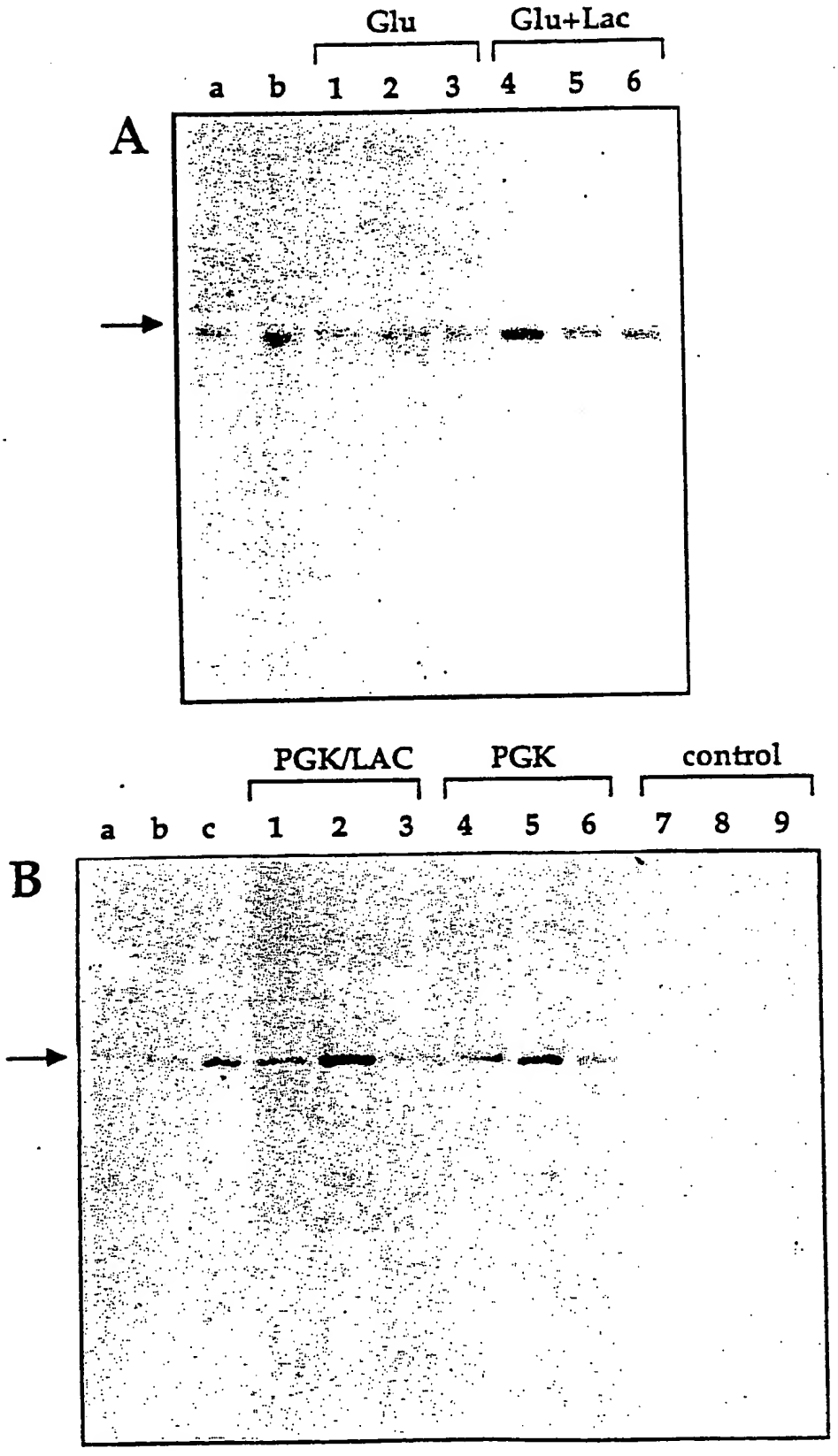


FIG_33

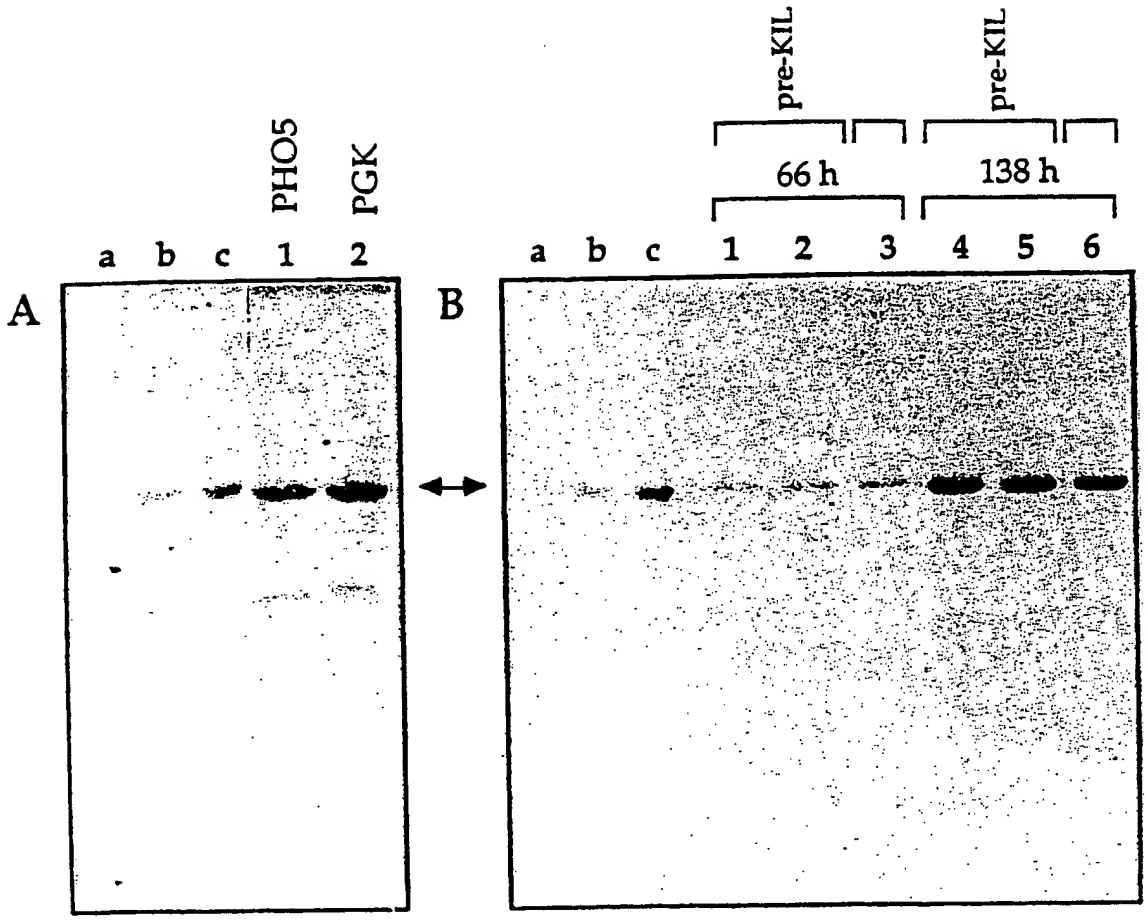


FIG_34

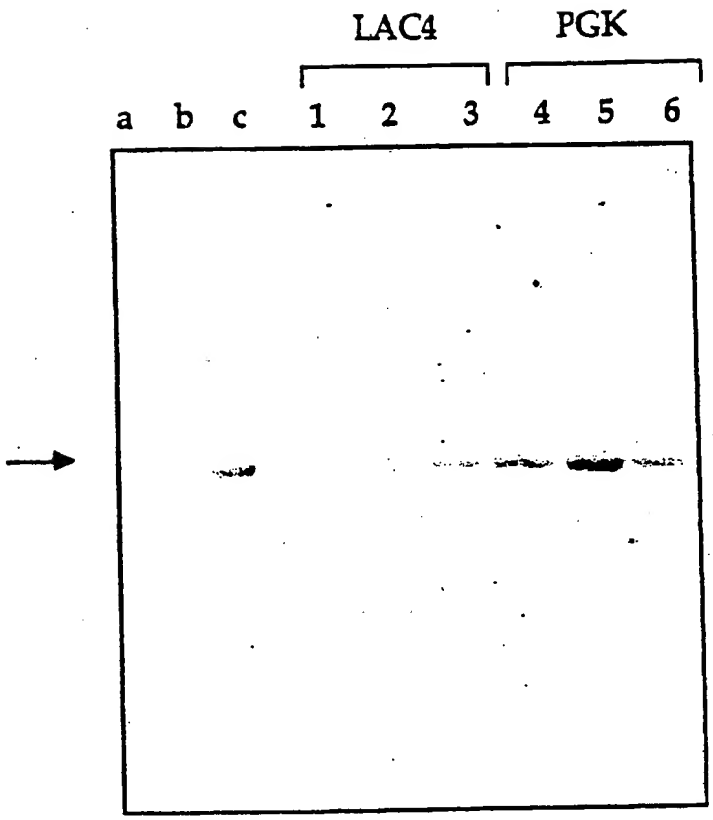


FIG_35

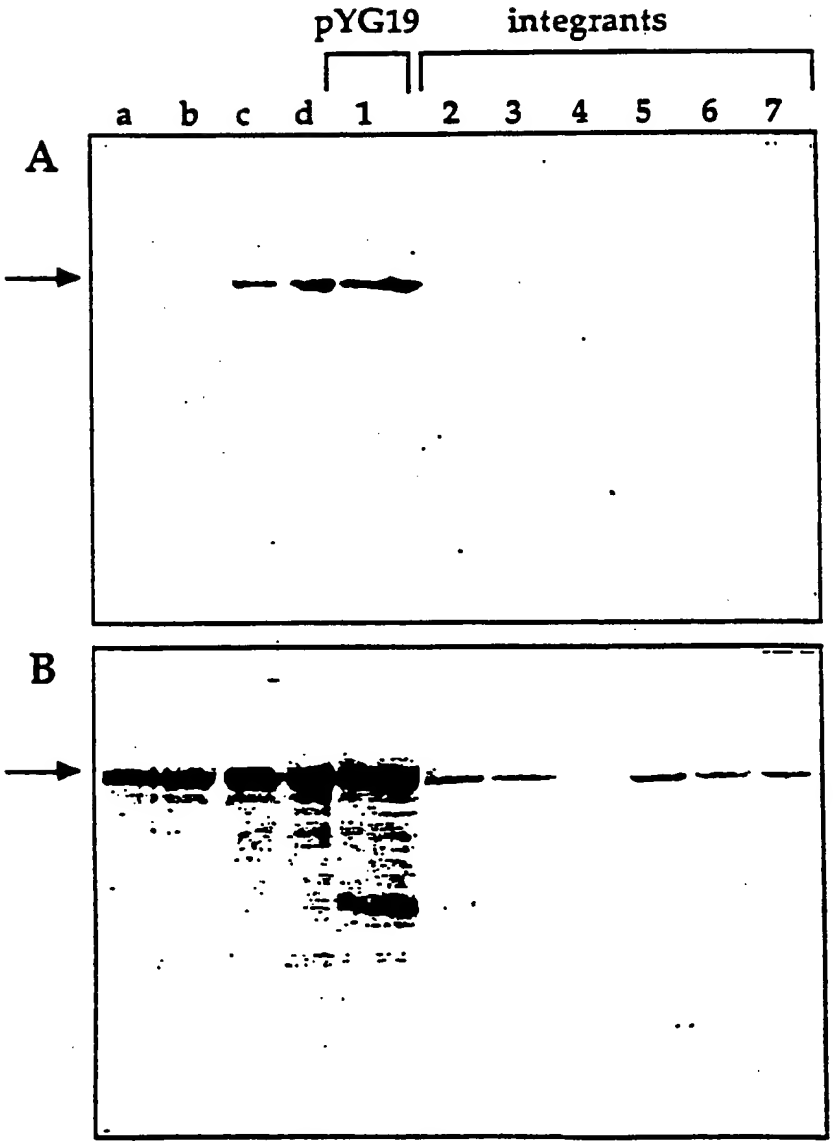


FIG_36

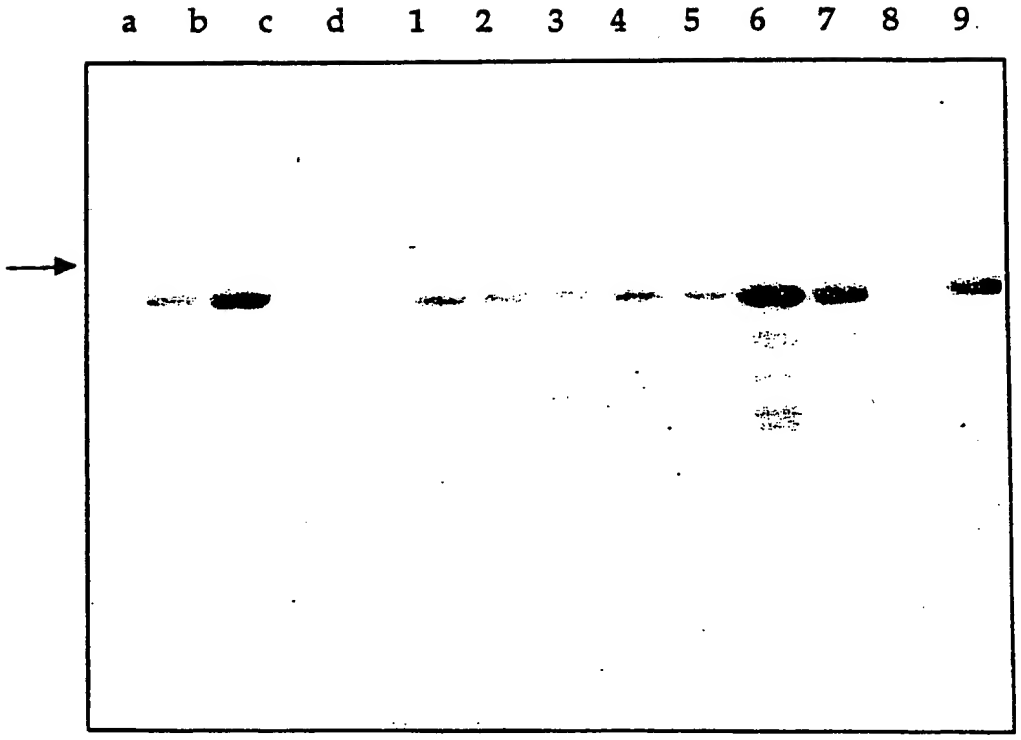


FIG_37

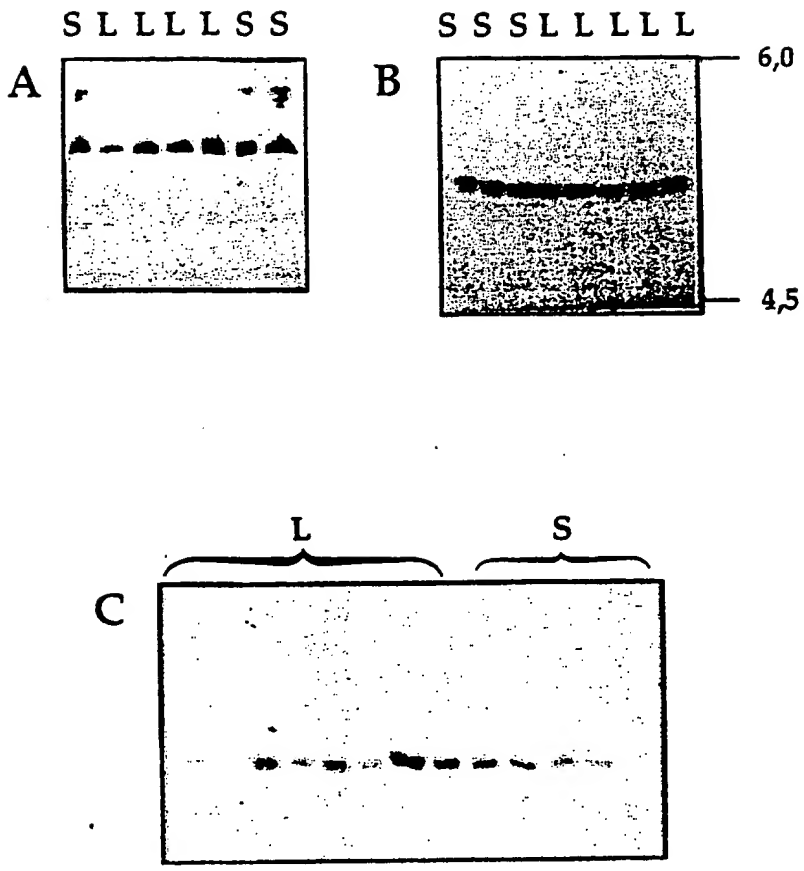


FIG_38

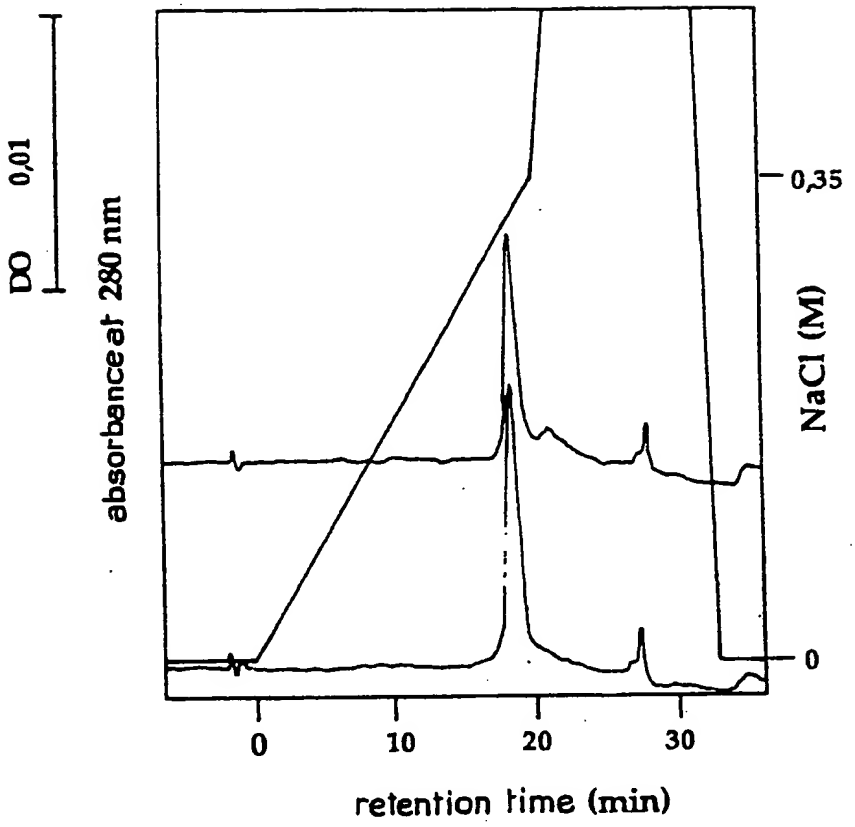

DO 0,01
absorbance at 280 nm
0,35
0
NaCl (M)
0
10
20
30
retention time (min)

FIG_39

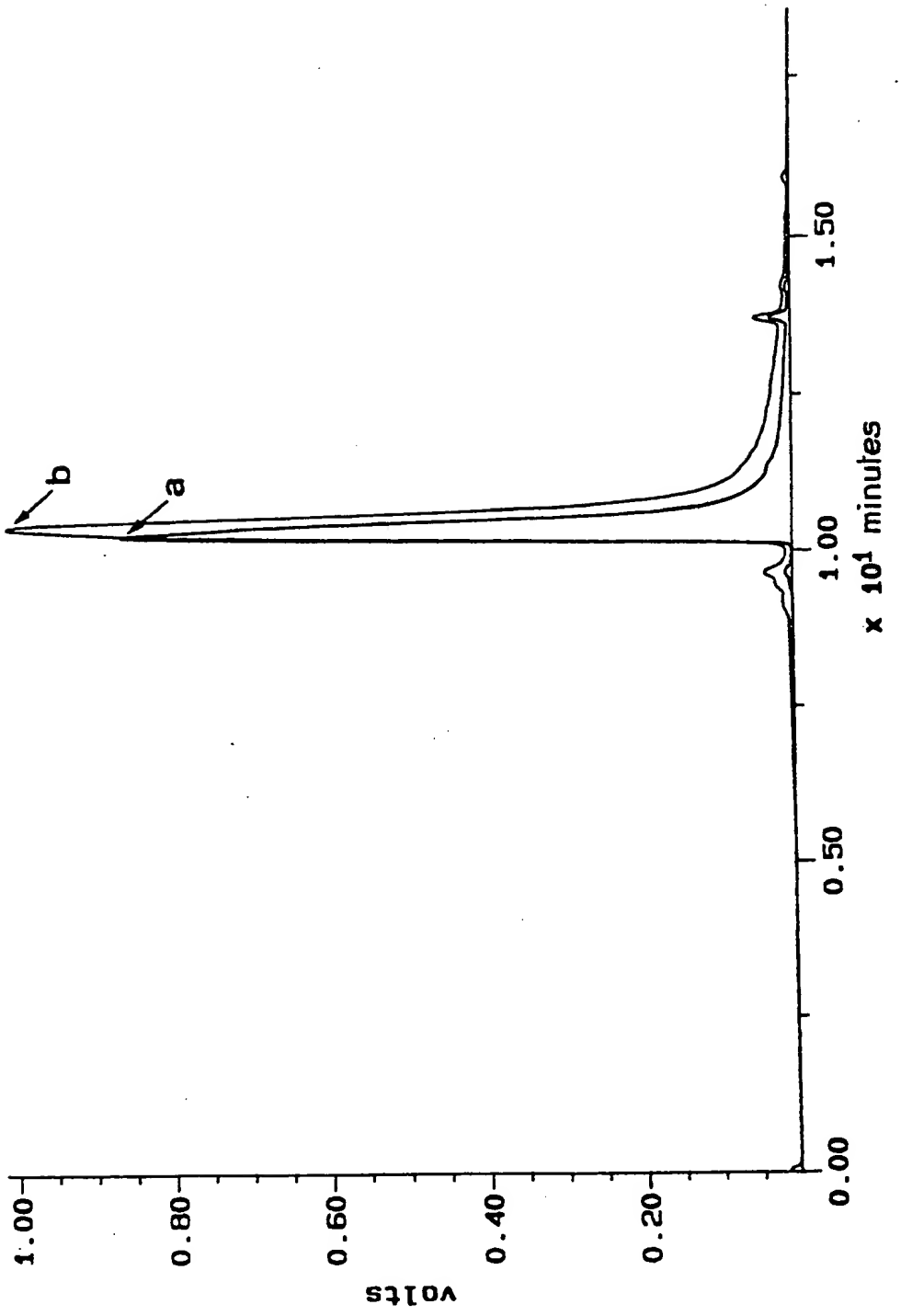


FIG_40

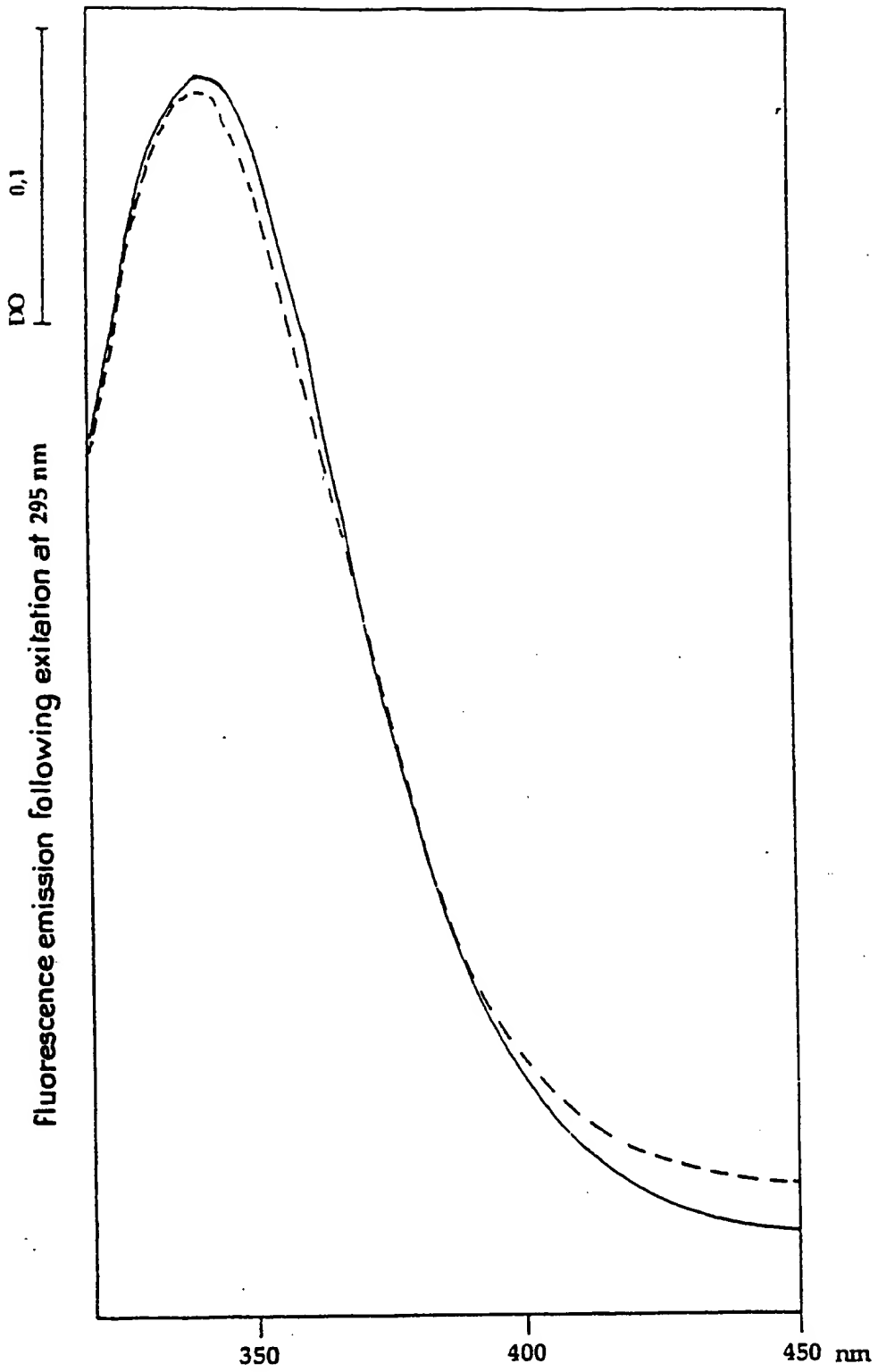

0,1
I.D
fluorescence emission following exitation at 295 nm
350
400
450 nm

FIG_41

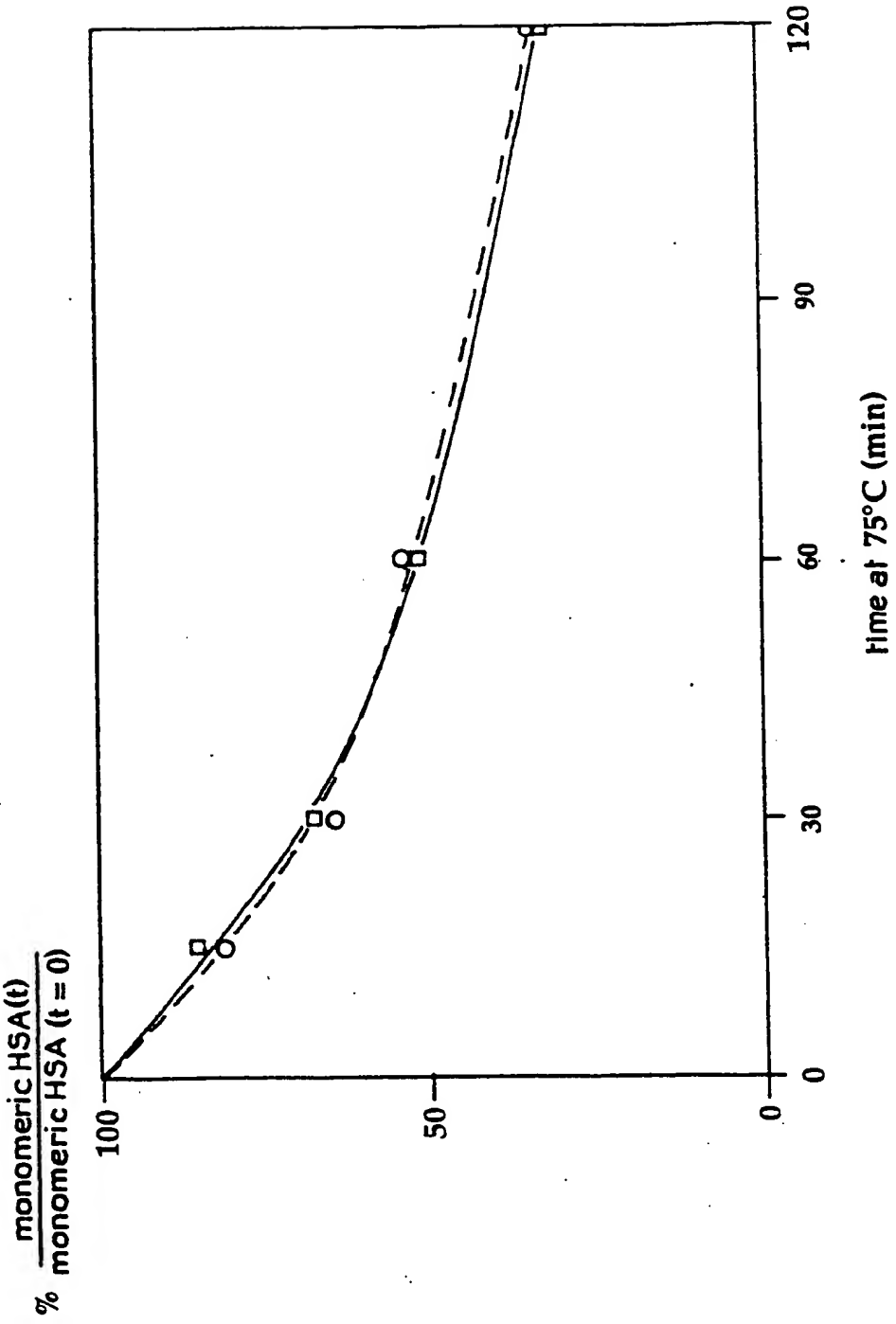

% monomeric HSA(t) / monomeric HSA (t = 0)
100
50
0
0
30
60
90
120
Time at 75°C (min)

FIG_42

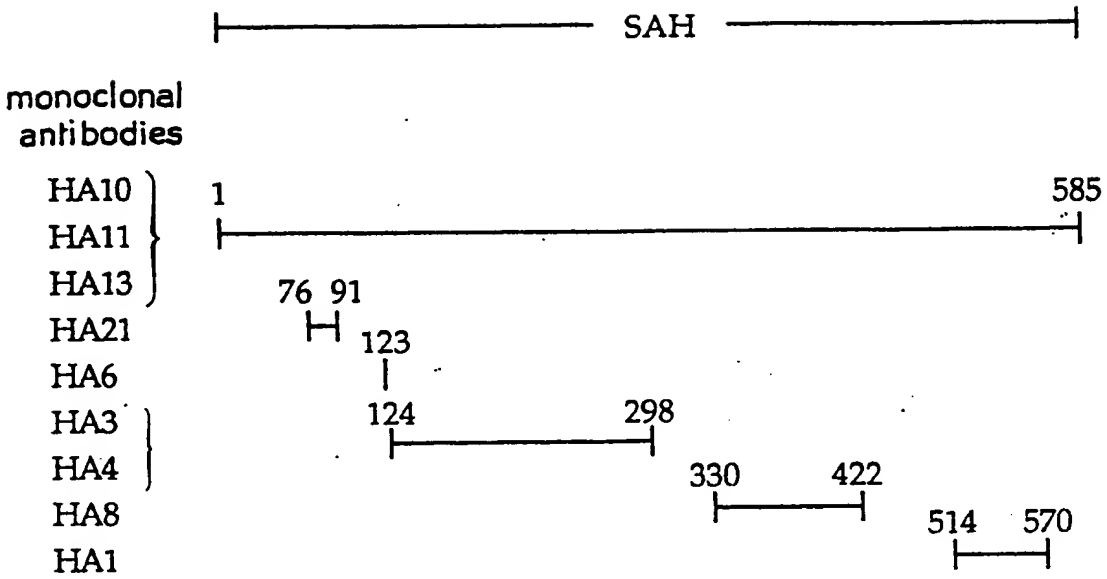

SAH
monoclonal antibodies
HA10
HA11
HA13
HA21
HA6
HA3
HA4
HA8
HA1
1
585
76
91
123
124
298
330
422
514
570

FIG_43

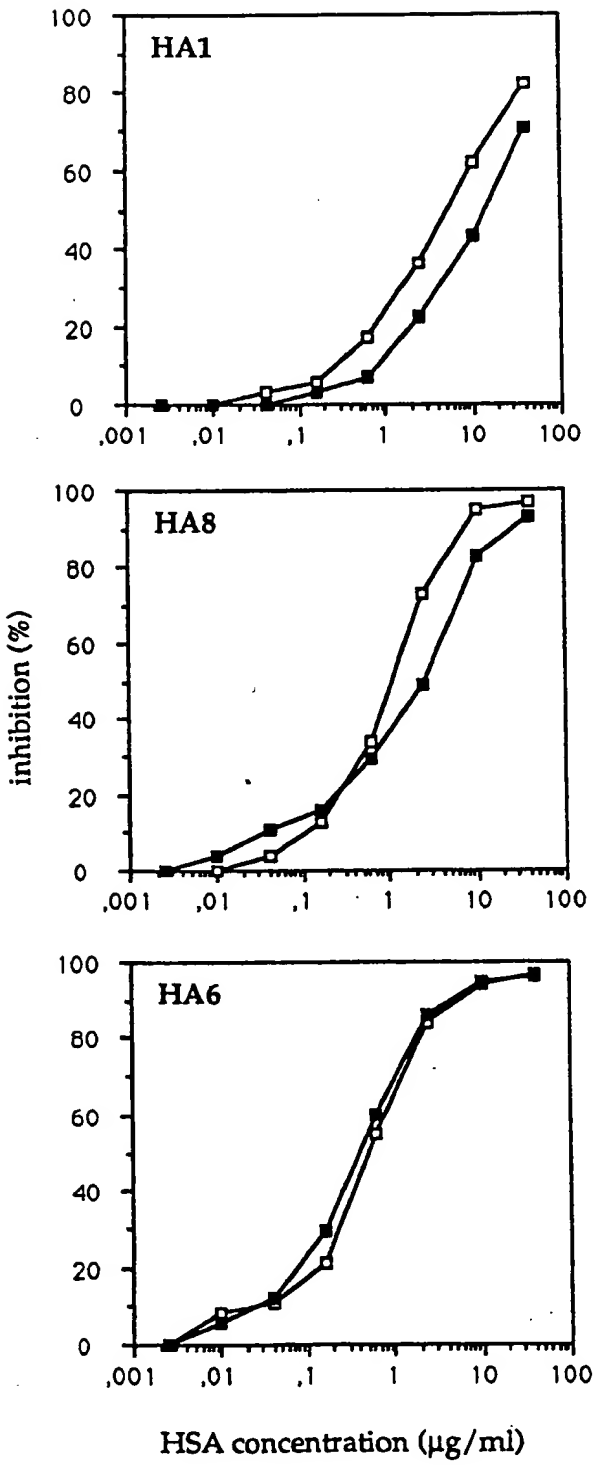

HA1
HA8
HA6
100
80
60
40
20
0
,001
,01
,1
1
10
100
inhibition (%)
HSA concentration (µg/ml)

FIG_44A

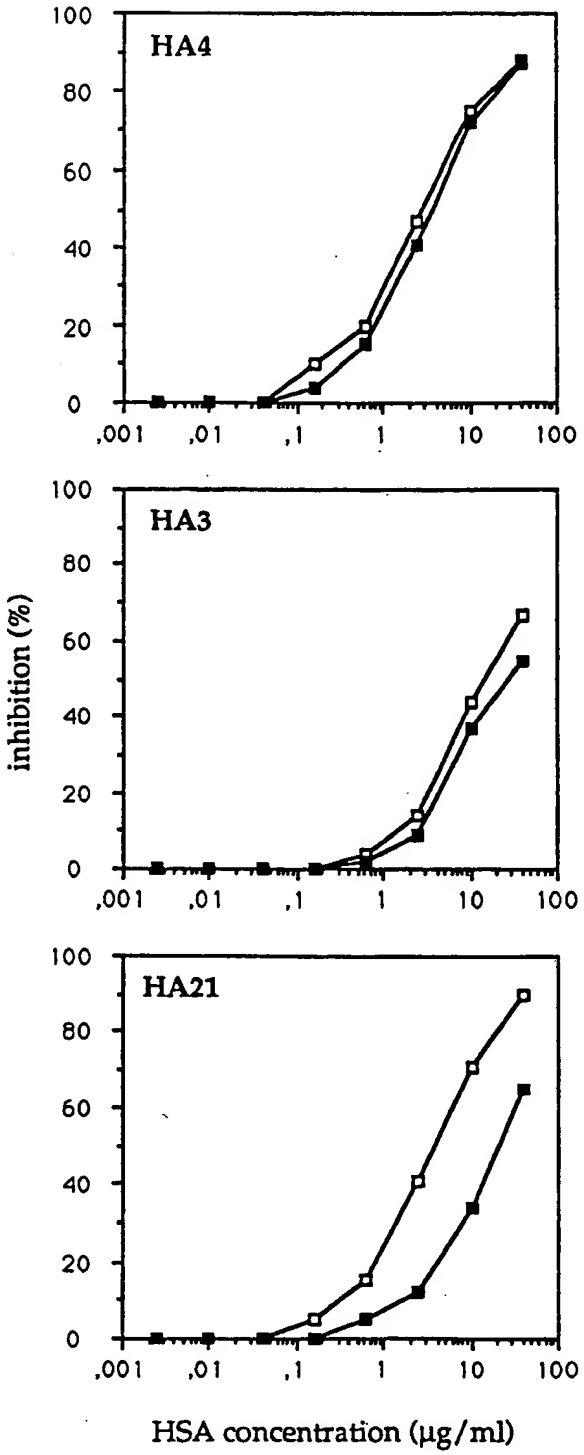

HA4
HA3
HA21
inhibition (%)
100
80
60
40
20
0
,001
,01
,1
1
10
100
HSA concentration (µg/ml)

FIG_44B

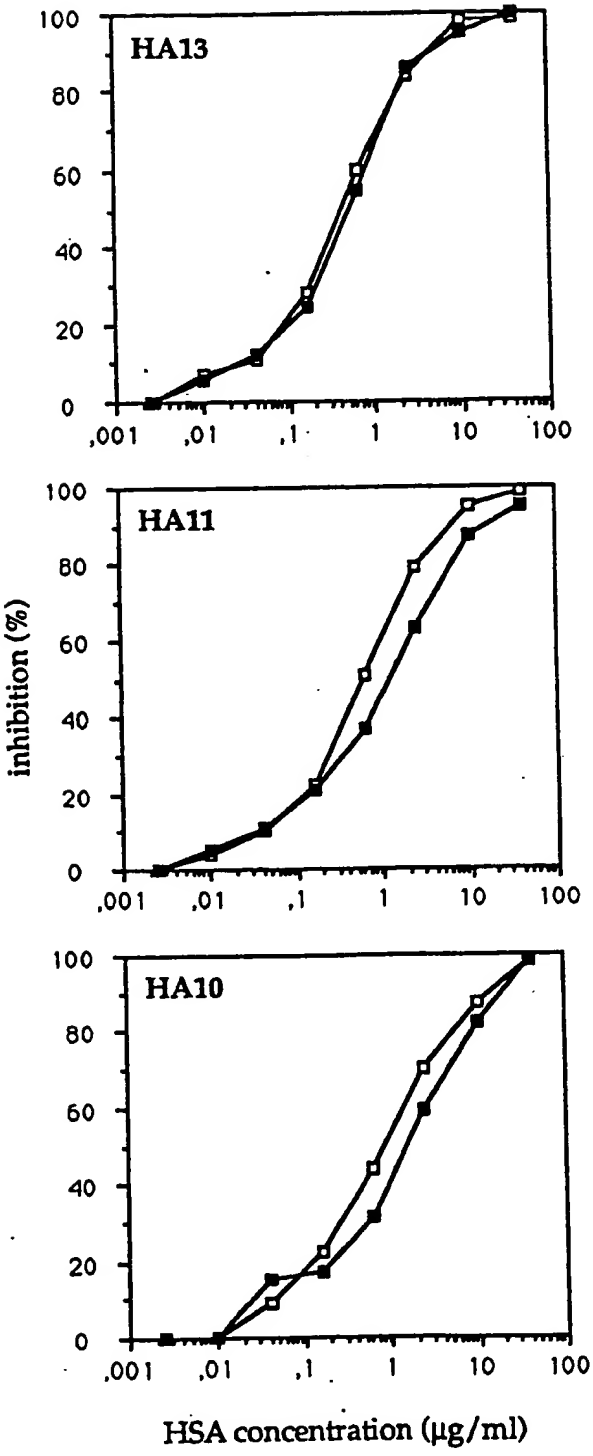

HA13
HA11
HA10
inhibition (%)
100
80
60
40
20
0
,001
,01
,1
1
10
100
HSA concentration (µg/ml)

FIG_44C

(EcoRI°) Met Asn Ile Phe Tyr Ile Phe Leu Phe Leu Leu Ser Phe Val Gln Gly Lys Arg

AATT ATG AAT ATA TTT TAC ATA TTT TTG TTT TTG CTG TCA TTC GTT CAA GGT AAA AG
TAC TTA TAT AAA ATG TAT AAA AAC AAA AAC GAC AGT AAG CAA GTT CCA TTT TCT TAA

EcoRI

EcoRI synthetic fragment encoding the killer toxin secretion signal

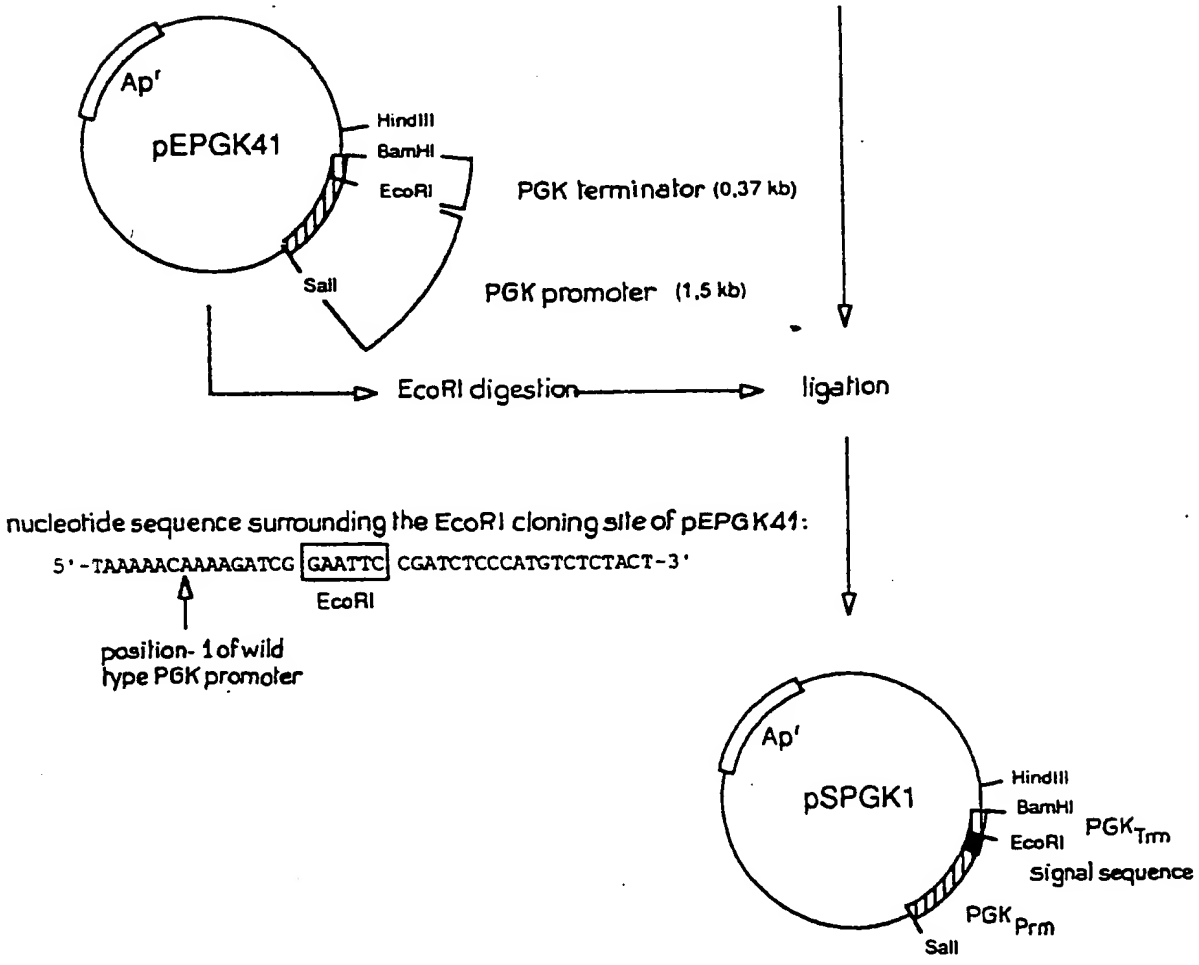


FIG. 45

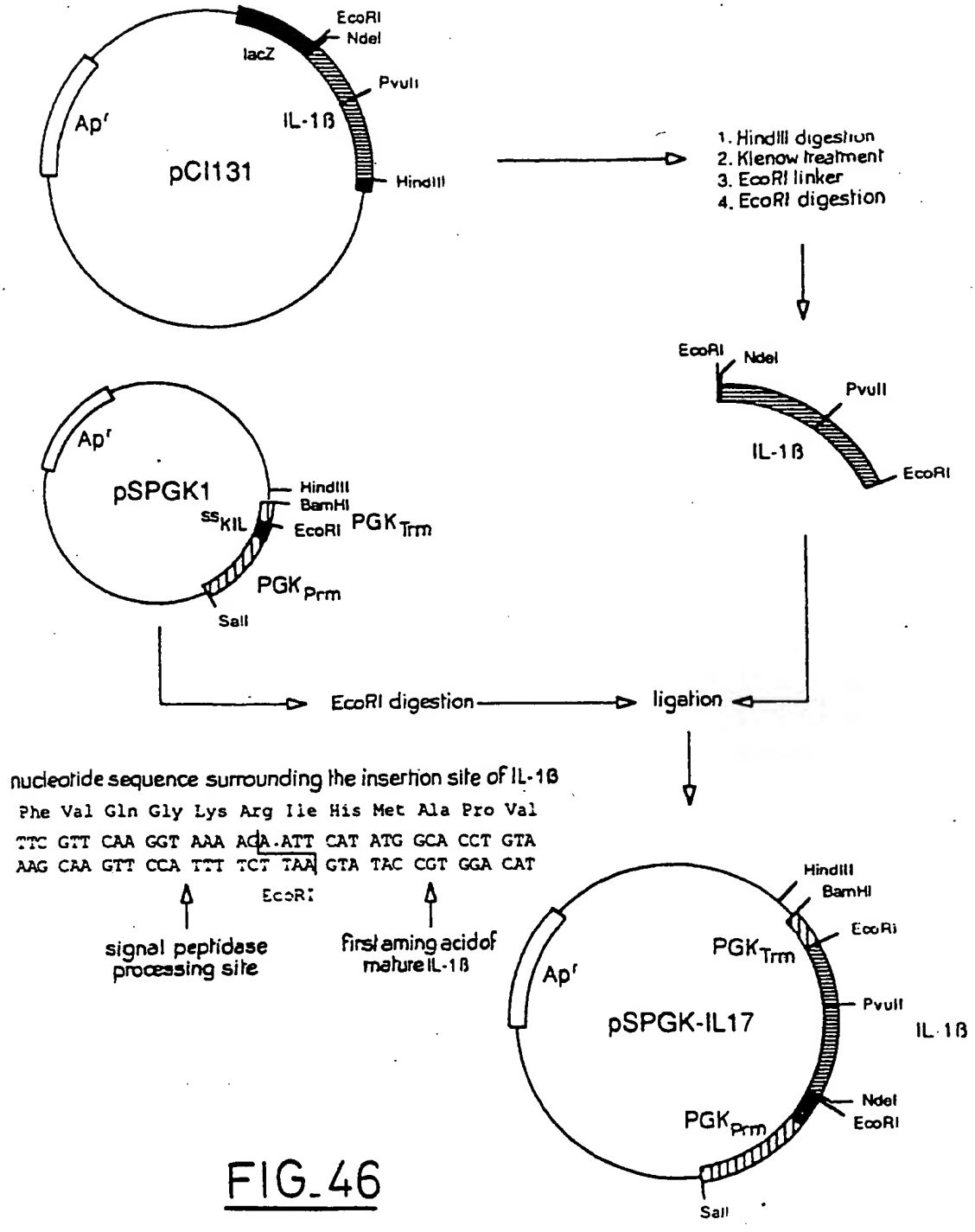

EcoRI
NdeI
lacZ
PvuII
Ap^r
IL-1β
pCI131
HindIII
1. HindIII digestion
2. Klenow treatment
3. EcoRI linker
4. EcoRI digestion
EcoRI
NdeI
PvuII
IL-1β
EcoRI
Ap^r
pSPGK1
HindIII
BamHI
ssKIL
EcoRI
PGK Trm
PGK Prm
SalI
EcoRI digestion
ligation
nucleotide sequence surrounding the insertion site of IL-1β
Phe Val Gln Gly Lys Arg Ile His Met Ala Pro Val
TTC GTT CAA GGT AAA AGA ATT CAT ATG GCA CCT GTA
AAG CAA GTT CCA TTT TCT TAA GTA TAC CGT GGA CAT
EcoRI
signal peptidase processing site
first amino acid of mature IL-1β
HindIII
BamHI
EcoRI
PGK Trm
Ap^r
PvuII
pSPGK-IL17
IL-1β
NdeI
EcoRI
PGK Prm
SalI

FIG_46

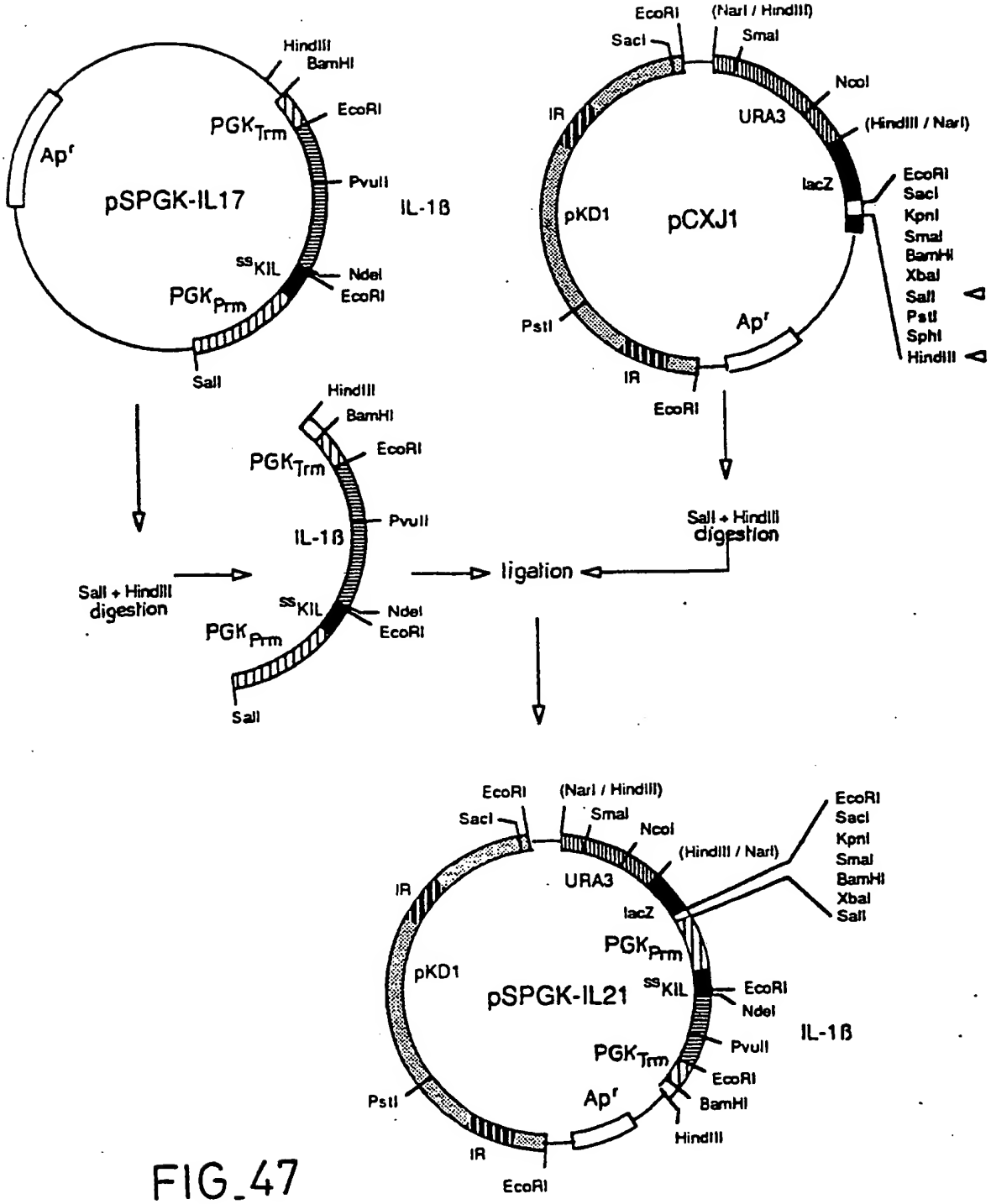

HindIII
BamHI
EcoRI
PGK Trm
Ap r
pSPGK-IL17
PvuII
IL-1ß
ss KIL
NdeI
EcoRI
PGK Prm
SalI
EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
IR
URA3
(HindIII / NarI)
lacZ
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
pKD1
pCXJ1
PstI
Ap r
IR
EcoRI
SalI + HindIII digestion
ligation
SalI + HindIII digestion
pSPGK-IL21

FIG_47

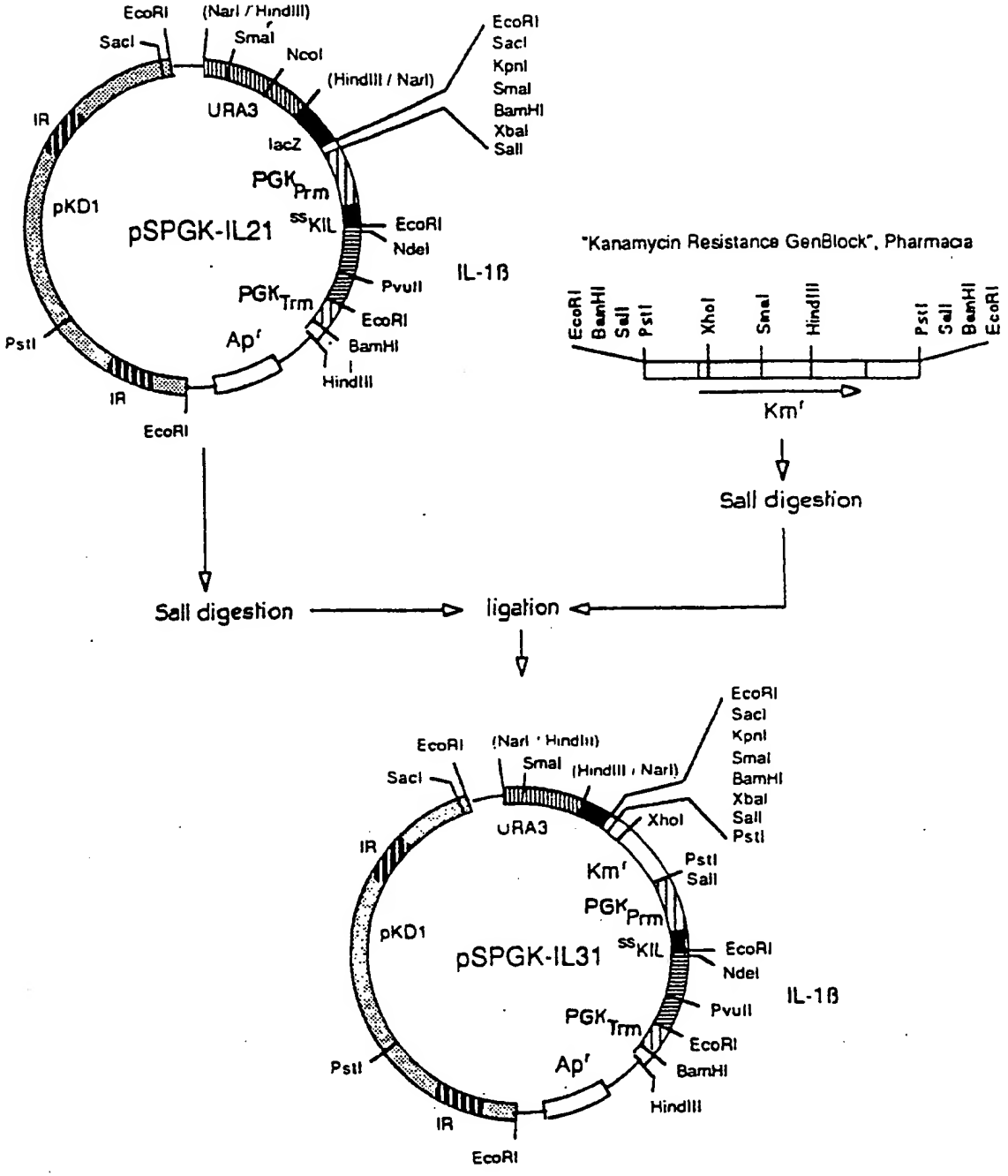

EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
(HindIII / NarI)
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
URA3
IR
lacZ
PGK Prm
pKD1
pSPGK-IL21
ssKIL
EcoRI
NdeI
IL-1β
PvuII
PGK Trm
EcoRI
PstI
Ap r
BamHI
HindIII
IR
EcoRI
"Kanamycin Resistance GenBlock", Pharmacia
EcoRI BamHI SalI PstI XhoI SmaI HindIII PstI SalI BamHI EcoRI
Km r
SalI digestion
SalI digestion
ligation
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
EcoRI
(NarI / HindIII)
SacI
SmaI
(HindIII / NarI)
XhoI
URA3
IR
PstI
SalI
Km r
PGK Prm
pKD1
pSPGK-IL31
ssKIL
EcoRI
NdeI
IL-1β
PvuII
PGK Trm
EcoRI
PstI
Ap r
BamHI
HindIII
IR
EcoRI

FIG_48

(EcoRI°) Met Asn Ile Phe Tyr Ile Phe Leu Phe Leu Leu Ser Phe Val Gln Gly Lys Arg

AATT ATG AAT ATA TTT TAC ATA TTT TTG TTT TTG CTG TCA TTC GTT CAA GGT AAA AG
TAC TTA TAT AAA ATG TAT AAA AAC AAA AAC GAC AGT AAG CAA GTT CCA TTT TCT TAA

EcoRI

synthetic EcoRI fragment coding for the killer toxin secretion signal

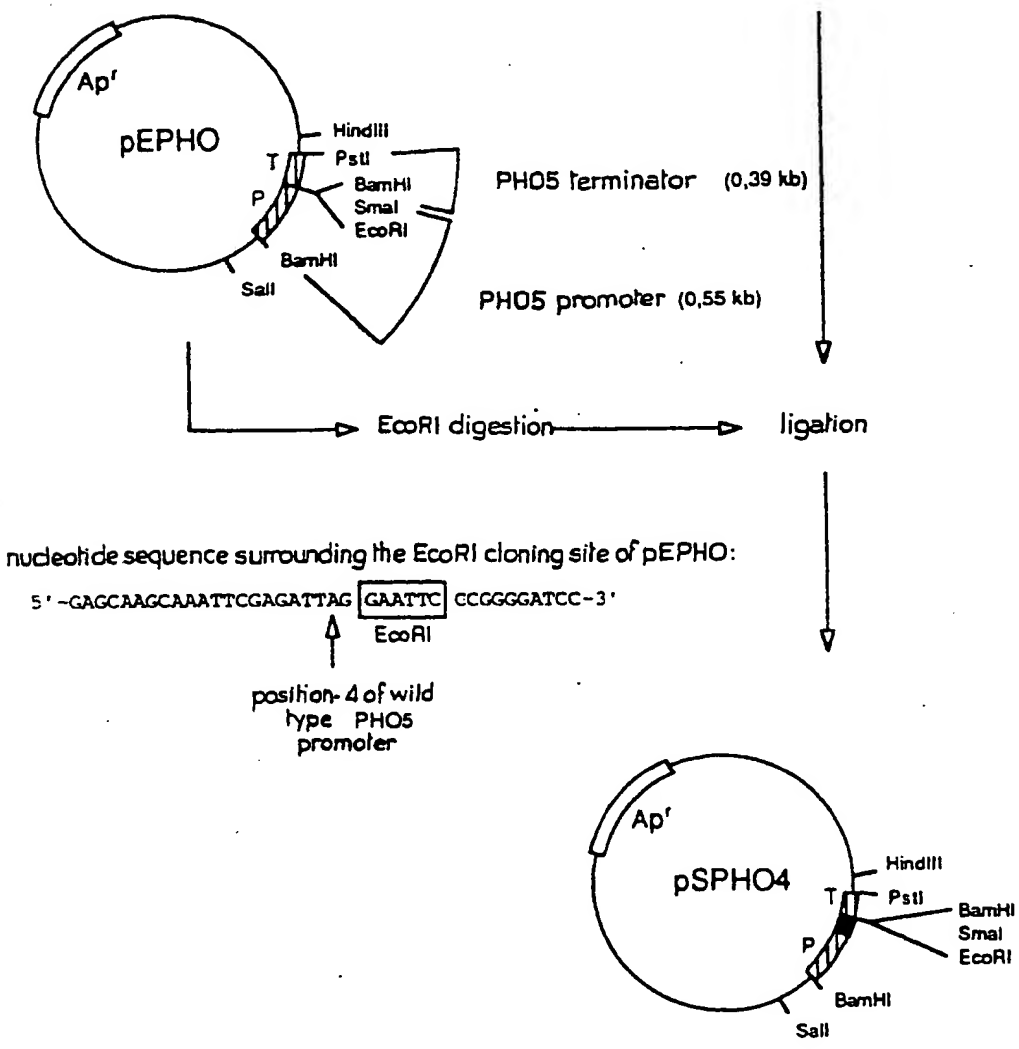


FIG_49

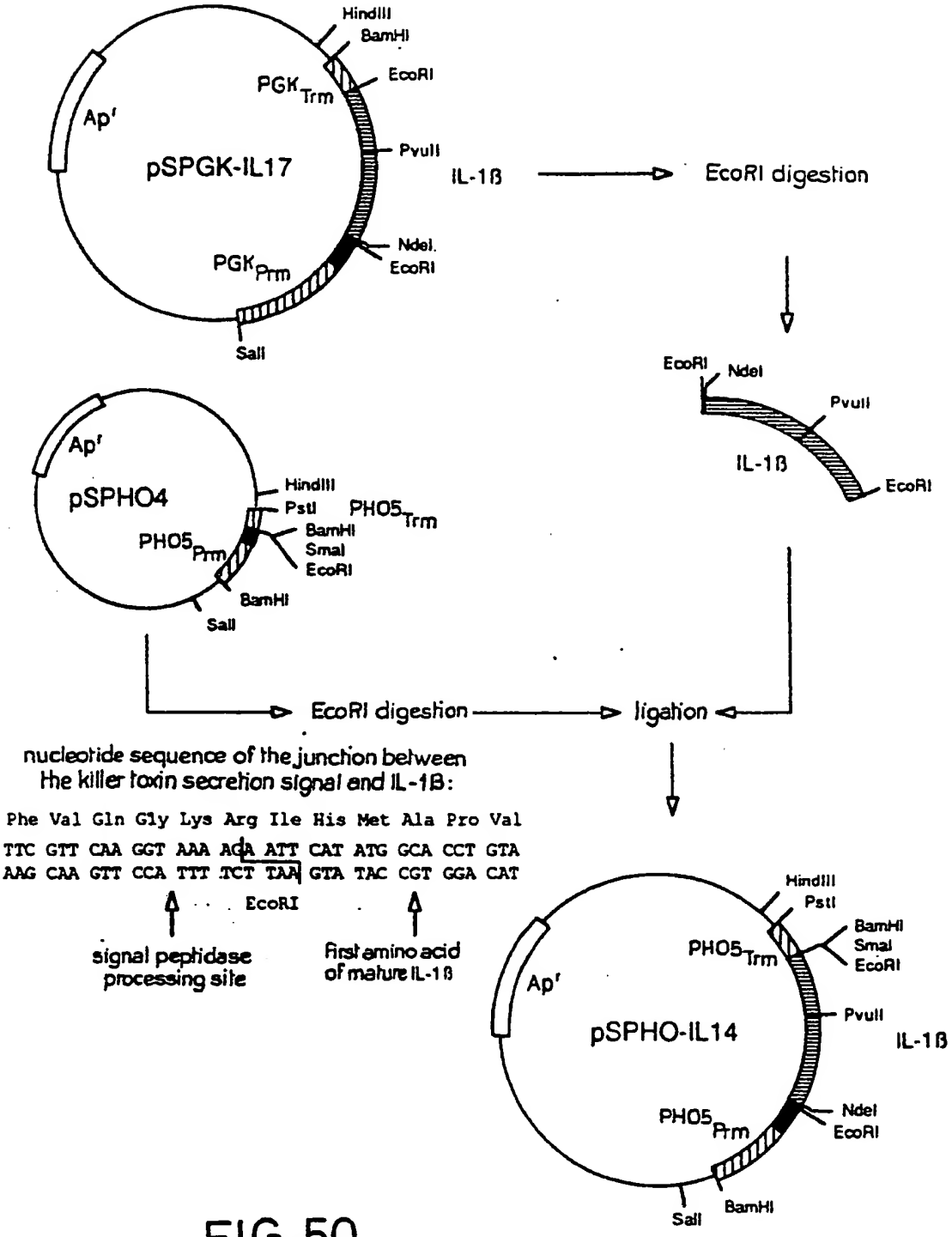

HindIII
BamHI
EcoRI
PGK Trm
Ap'
pSPGK-IL17
PvuII
IL-1β
EcoRI digestion
NdeI.
EcoRI
PGK Prm
SalI
EcoRI
NdeI
PvuII
IL-1β
EcoRI
Ap'
pSPHO4
HindIII
PstI
PHO5 Trm
BamHI
SmaI
EcoRI
PHO5 Prm
BamHI
SalI
EcoRI digestion
ligation
nucleotide sequence of the junction between the killer toxin secretion signal and IL-1B:
Phe Val Gln Gly Lys Arg Ile His Met Ala Pro Val
TTC GTT CAA GGT AAA AGA ATT CAT ATG GCA CCT GTA
AAG CAA GTT CCA TTT TCT TAA GTA TAC CGT GGA CAT
EcoRI
signal peptidase processing site
first amino acid of mature IL-1β
HindIII
PstI
BamHI
SmaI
EcoRI
PHO5 Trm
Ap'
pSPHO-IL14
PvuII
IL-1β
PHO5 Prm
NdeI
EcoRI
SalI
BamHI

FIG_50

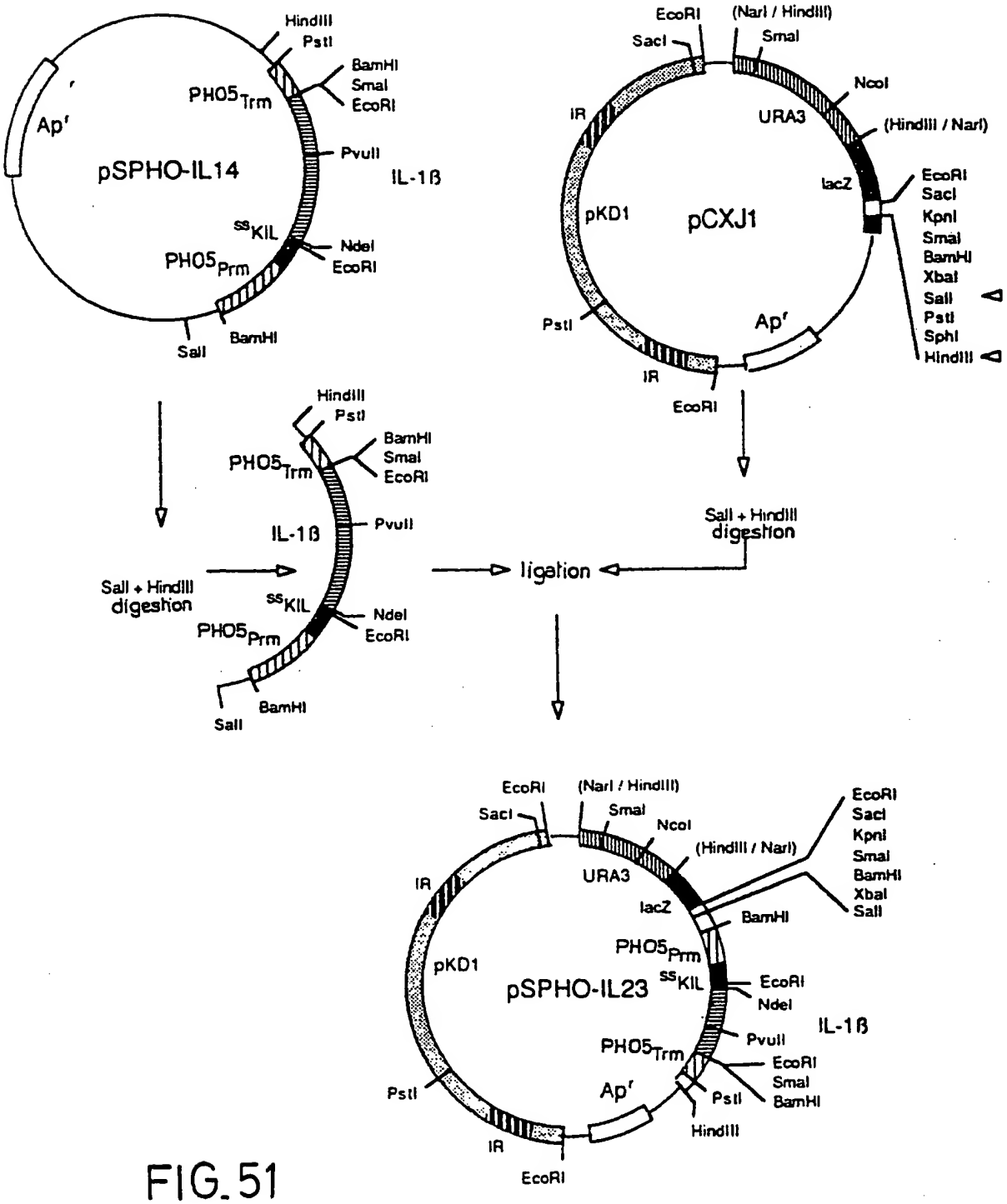

HindIII
PstI
BamHI
SmaI
EcoRI
PHO5Trm
Ap'
pSPHO-IL14
PvuII
IL-1ß
ssKIL
NdeI
EcoRI
PHO5Prm
SalI
BamHI
EcoRI
(NarI / HindIII)
SacI
SmaI
NcoI
IR
URA3
(HindIII / NarI)
lacZ
pKD1
pCXJ1
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
PstI
Ap'
IR
EcoRI
SalI + HindIII digestion
ligation
SalI + HindIII digestion
pSPHO-IL23

FIG. 51

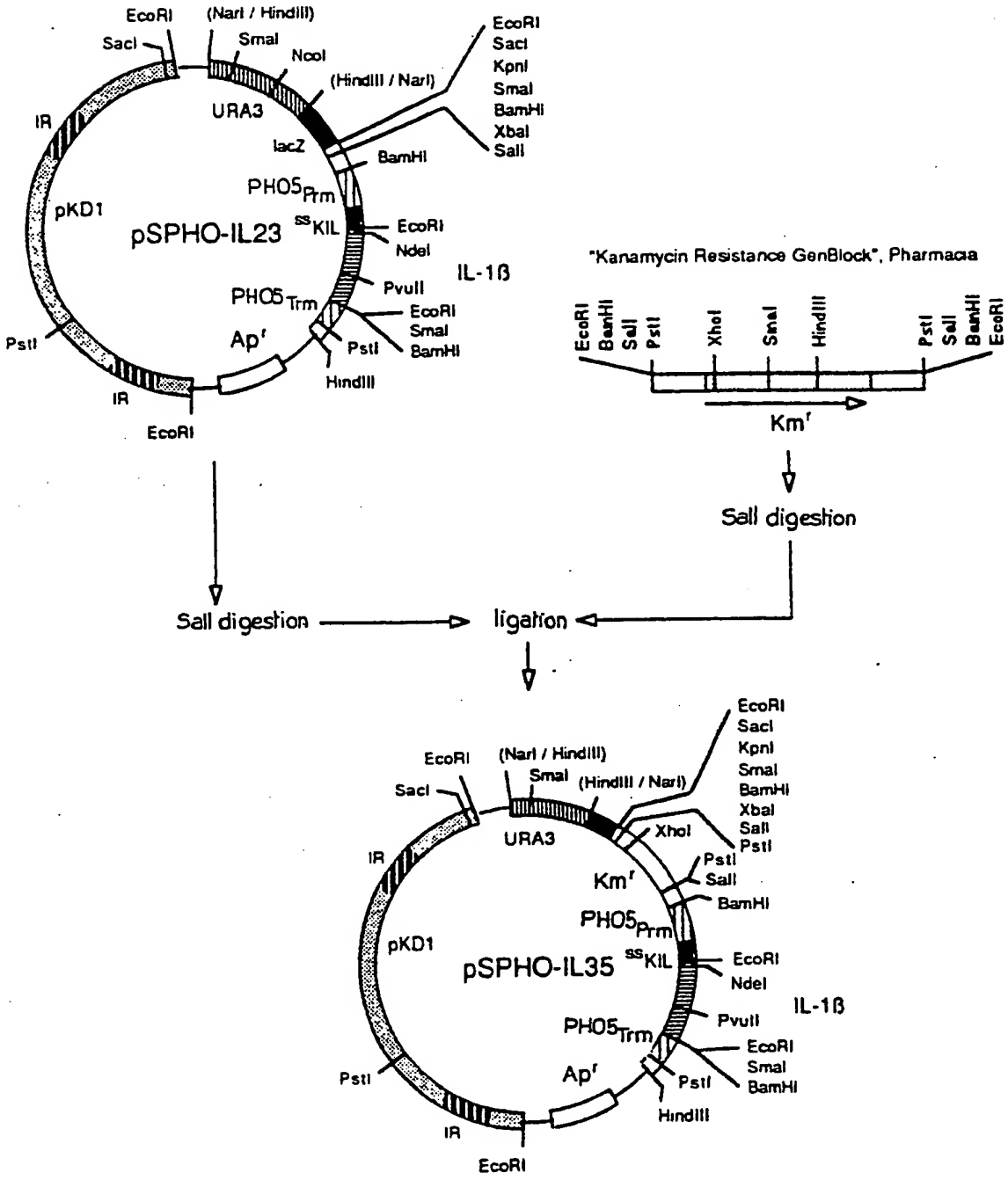

EcoRI
SacI
(NarI / HindIII)
SmaI
NcoI
(HindIII / NarI)
URA3
lacZ
BamHI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
IR
pKD1
PHO5Prm
pSPHO-IL23
ssKIL
EcoRI
NdeI
IL-1β
PvuII
PHO5Trm
EcoRI
SmaI
BamHI
Ap^r
PstI
HindIII
PstI
IR
EcoRI
"Kanamycin Resistance GenBlock", Pharmacia
EcoRI
BamHI
SalI
PstI
XhoI
SmaI
HindIII
PstI
SalI
BamHI
EcoRI
Km^r
SalI digestion
SalI digestion
ligation
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
EcoRI
(NarI / HindIII)
SmaI
(HindIII / NarI)
SacI
XhoI
URA3
IR
PstI
SalI
Km^r
BamHI
PHO5Prm
pKD1
pSPHO-IL35
ssKIL
EcoRI
NdeI
IL-1β
PvuII
PHO5Trm
EcoRI
SmaI
BamHI
Ap^r
PstI
PstI
HindIII
IR
EcoRI

FIG.52

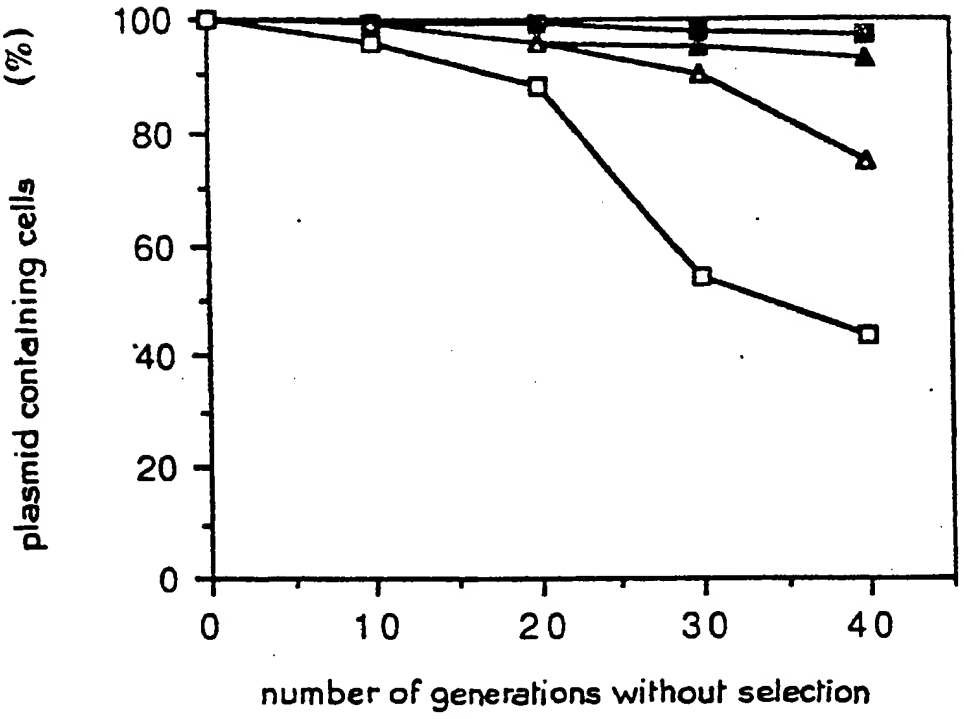

plasmid containing cells (%)
100
80
60
40
20
0
0
10
20
30
40
number of generations without selection

FIG_53

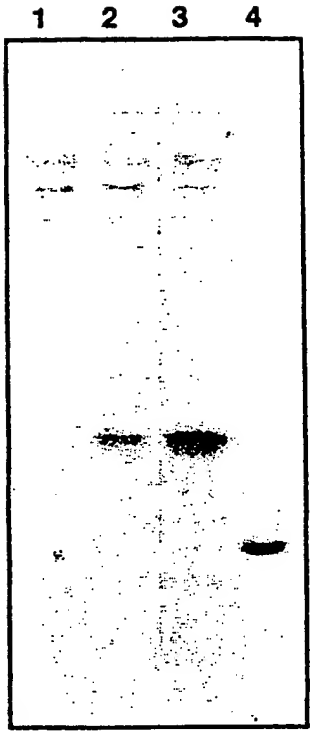


FIG_54

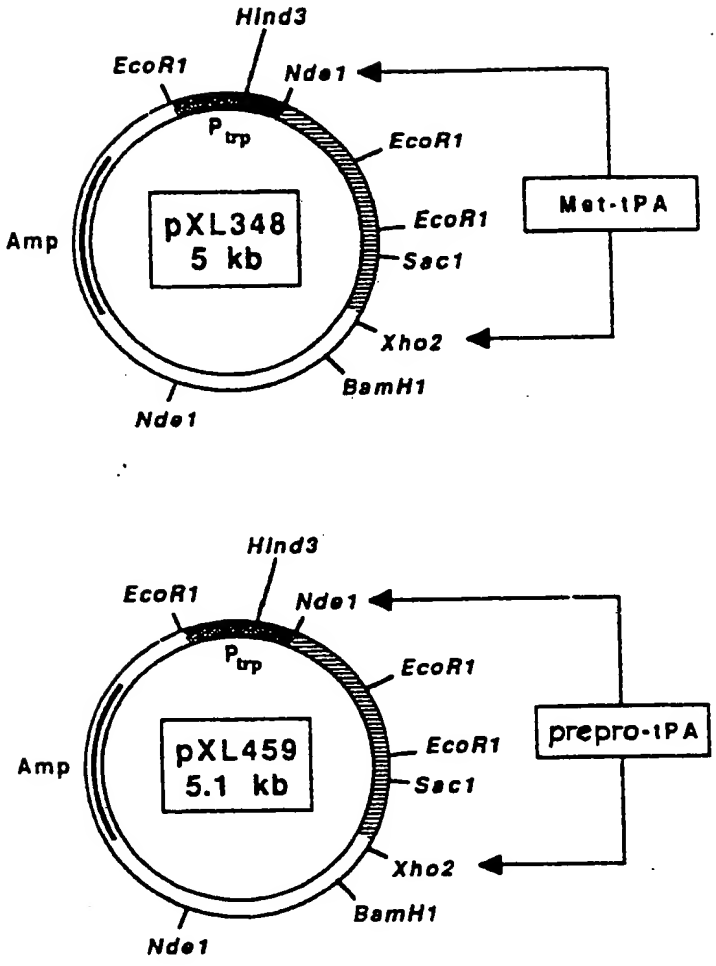

HInd3
EcoR1
Nde1
P trp
EcoR1
pXL348
5 kb
Amp
EcoR1
Sac1
Met-tPA
Xho2
BamH1
Nde1
HInd3
EcoR1
Nde1
P trp
EcoR1
pXL459
5.1 kb
Amp
EcoR1
Sac1
prepro-tPA
Xho2
BamH1
Nde1

FIG.55

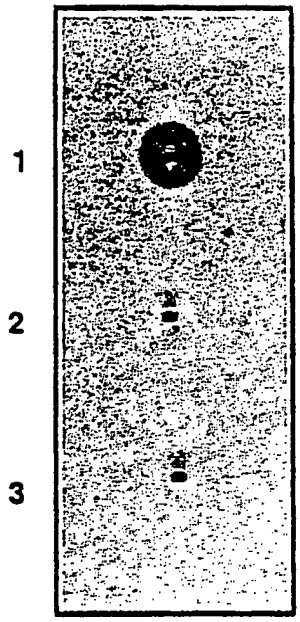


FIG_56

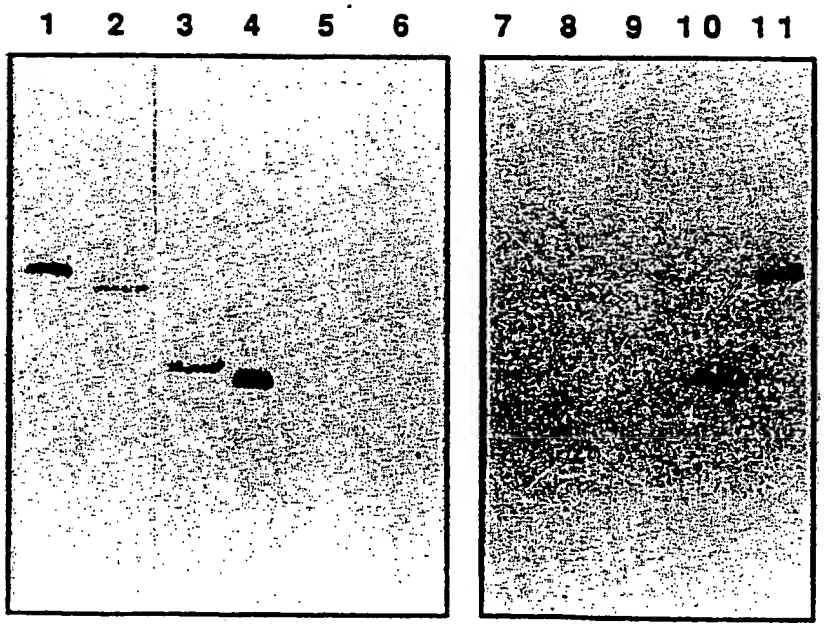


FIG_57